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(54) Title: COMPOSITIONS AND METHODS FOR INCREASING ANIMAL SIZE AND GROWTH RATE

(57) Abstract: The present invention relates to germ line and somatic cells comprising a mutant $p27_{kip1}$ protein lacking a Cdk2 phosphorylation site. Also provided are transgenic animals and methods of making such transgenic animals which have increased size and/or growth rate.

COMPOSITIONS AND METHODS FOR INCREASING ANIMAL SIZE AND

GROWTH RATE

RELATED APPLICATIONS

10   This application claims priority to U.S. provisional application number 60/352,391,
filed on January 28, 2003, the disclosure of which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

15   Animal cells have both a proliferating phase and a quiescent phase. Cells can shift
from the proliferating phase to the quiescent phase during a brief window in the cell cycle.
Depending on their position in the cell cycle, cells deprived of mitogens such as those
present in serum can undergo immediate cell cycle arrest; or they can complete the current
mitotic cycle and arrest in the next cell cycle. The transition from mitogen-dependence to
20   mitogen-independence occurs in mid- to late-G1 phase of the cell cycle. Anti-mitogenic
signals can cause the cell cycle to arrest at a kinetically common point. In particular, in early
G1, cells can exit the cell cycle. Cell cycle commitment (autonomy from mitogenic signals)
occurs in mid-G1.

        The transition of cells through G1 and entry into S phase requires the action of
25   cyclin-dependent kinases (Cdks). Growth inhibitory signals have been shown to prevent
activation of these Cdks during G1 (Serrano *et al.*, *Nature* 366:704-07 (1993); Hannon and
Beach, *Nature* 371:257-61 (1994); Xiong *et al.*, *Nature* 366:701-04 (1993); Polyak *et al.*,
Cell 78:59-66 (1994); Lee *et al.*, *Genes & Development* 9:639-49 (1995); Koff *et al.*, *Science*
260:536-39 (1993)). The catalytic activity of Cdks is known to be regulated by two general
30   mechanisms: protein phosphorylation and association with regulatory subunits (Gould *et al.*,
EMBO J. 10:3297-309 (1991); Solomon *et al.*, *EMBO J.* 12:3133-42 (1993); Solomon *et al.*,

Mol. Biol. Cell 3:13-27 (1992); Jeffrey *et al.*, *Nature* 376:313-20 (1995); Morgan, *Nature* 374:131-34 (1995)). Among the regulatory subunits, the association of Cdks with inhibitory CKI subunits (Cyclin-dependent Kinase Inhibitors) has been most closely correlated with the effect of mitogen depletion on cell proliferation and Cdk activity.

5   The CKI directly implicated in mitogen-dependent Cdk regulation is p27[Kip1] (Polyak *et al.*, *Cell* 78:59-66 (1994); Toyoshima and Hunter, *Cell* 78:67-77 (1994)). Wildtype p27[Kip1] protein accumulates to high levels in quiescent cells, and is rapidly destroyed after quiescent cells are re-stimulated with specific mitogens (Nourse *et al.*, *Nature* 372:570-73 (1994); Kato *et al.*, *Cell* 79:487-96 (1994)). The destruction of p27[Kip1] is controlled by

10   phosphorylation of p27[Kip1] at threonine 187 (T187). T187 is phosphorylated by Cdk2 to create a binding site for a Skp2-containing ubiquitin-protein ligase known as the Skp1-cullin-F-box protein ligase (SCF) (Feldman *et al.*, *Cell* 91:221-30 (1997); Bai *et al.*, *Cell* 86:263-74 (1996); Skowyra *et al.*, *Cell* 91:209-19 (1997)). Ubiquitination of p27[Kip1] by the SCF then results in p27[Kip1] degradation by the proteosome (Sutterluty *et al.*, *Nature Cell*

15   *Biol.* 1:207-14 (1999); Rolfe *et al.*, *J. Mol. Med.* 75:5-17 (1997); Carrano *et al.*, *Nature Cell Biol.* 1:193-99 (1999); Tsvetkov *et al.*, *Curr. Biol.* 9:661-64 (1999)).

The destruction of p27[Kip1] was thought to be required for entry into S phase. Moreover, constitutive expression of p27[Kip1] in cultured cells causes the cell cycle to arrest in G1 (Polyak, *supra*; Toyoshima and Hunter, *supra*). Thus, based on these observations, it

20   was expected that cells harboring a null allele of p27[Kip1] would arrest G1. It was surprising, therefore, that animals harboring a null allele of the p27[Kip1] gene survived. Indeed, such animals were larger than normal (increased animal size) and without apparent gross morphologic abnormalities. (Fero *et al.*, *Cell* 85:733-44 (1996); U.S. Patent No. 5,958,769; the disclosures of which are incorporated by reference herein.) The advantages of producing

25   larger animals are readily apparent, and include increase meat, milk and/or egg production.

Decreased levels of p27[Kip1] in animals, however, cause certain minor defects, such as an ovulatory defect, and resulting female sterility, increased pituitary tumorigenesis and disrupted retinal architecture. (Fero *et al.*, *supra*.) These defects can interfere with some uses of such animals. Thus, there is a need for alternative mutant alleles of p27[Kip1], and of

30   methods of using such mutant alleles, that promote increased animal size or growth rate without these side effects.

SUMMARY OF THE INVENTION

The present invention relates to nucleic acids encoding a mutant $p27^{Kip1}$ protein that lacks a Cdk2 phosphorylation site, and to cells harboring mutant $p27^{Kip1}$ genes. In related aspects, transgenic cells and transgenic animals are provided that have one or more mutant

5      $p27^{Kip1}$ genes encoding protein that lacks a Cdk2 phosphorylation site.

In one aspect, isolated transgenic cells are provided comprising a mutant $p27^{Kip1}$ gene lacking a Cdk2 phosphorylation site. The mutant $p27^{Kip1}$ gene encodes a mutant $p27^{Kip1}$ protein having a longer half-life in S phase than wildtype $p27^{Kip1}$ polypeptide. In certain embodiments, the mutant $p27^{Kip1}$ polypeptide can inhibit Cdk2 *in vitro* kinase activity. In an

10   embodiment, the mutant $p27^{Kip1}$ polypeptide is $p27^{T187A}$.

The mutant $p27^{Kip1}$ gene can be located, for example, at an endogenous $p27^{Kip1}$ locus; the endogenous locus can be heterozygous or homozygous for the mutant $p27^{Kip1}$ gene. The transgenic cell can be, for example, a primordial germ cell, oocyte, egg, spermatocyte, sperm cell, fertilized egg, zygote, embryonic stem cell, or somatic cell. The transgenic cell can also

15   be progeny of any of these.

In another aspect, non-human, transgenic animals are provided which comprise a nucleic acid sequence encoding a mutant $p27^{Kip1}$ protein lacking a Cdk2 phosphorylation site. In an embodiment, the mutant $p27^{Kip1}$ protein is $p27^{T187A}$. The transgenic animal can be, for example, a primate, mammal, bovine, porcine, ovine, equine, avian, rodent, fowl,

20   piscine, or crustacean. In certain embodiments, the transgenic animal is a farm animal, such as a chicken, cow, bull, horse, pig, sheep, goose or duck.

In a related aspect, a transgenic, non-human animal is provided whose genome comprises a $p27^{Kip1}$ gene and expresses a mutant $p27^{Kip1}$ polypeptide having a longer half-life in S phase than wildtype p27 polypeptide. Expression of the mutant $p27^{Kip1}$ polypeptide

25   results in increased size or growth rate of the animal. The transgenic animal, can be, for example, a primate, mammal, bovine, porcine, ovine, equine, avian, rodent, fowl, piscine, or crustacean. In certain embodiments, the transgenic animal is a farm animal, such as a chicken, cow, bull, horse, pig, sheep, goose or duck.

Methods of increasing the size or growth rate of a non-human, transgenic animal are

30   also provided. Such methods generally include stably introducing into a genome of an animal cell a mutant $p27^{Kip1}$ gene lacking a Cdk2 phosphorylation site; and producing an animal from the animal cell. In an embodiment, the method further includes transferring a nucleus from the animal cell into a second cell from which an animal can be reconstituted;

and allowing the second cell to develop into an immature animal. The immature animal typically is larger than an immature animal not having the mutant $p27^{Kip1}$ gene. The second cell, can be, for example, an enucleated fertilized egg.

In another embodiment, the mutant $p27^{Kip1}$ gene can be homologously integrated at an endogenous $p27^{Kip1}$ locus in the animal cell. The mutant $p27^{Kip1}$ gene can be homologous or heterologous to the animal cell, and can be integrated at an endogenous $p27^{Kip1}$ locus or at a non-$p27^{Kip1}$ locus. The mutant $p27^{Kip1}$ gene can encode, for example, $p27^{T187A}$ protein.

The animal cell can be, for example, a germ cell, a totipotent cell, a stem cell, an embryonic stem cell, a pluripotent stem cell, a fetal cell, a primordial germ cell, an oocyte, an egg, a spermatocyte, a sperm cell, a fertilized egg, a zygote, a blastomere, or a somatic cell. The animal cell can be a vertebrate cell, such as, for example, from a primate, mammal, bovine, porcine, ovine, equine, avian, rodent, fowl, piscine, or crustacean. Exemplary animals include a chicken, hen, rooster, cow, bull, duck or goose.

Mutant genes can be introduced into cells by electroporation, microinjection, lipofection, transfection, biolistics, and the like. The mutant $p27^{Kip1}$ genes can be introduced alone or as part of an expression cassette that includes, for example, a heterologous promoter operably associated with an open reading frame encoding a mutant $p27^{Kip1}$ gene operably associated with a polyadenylation sequence. The expression cassette can also optionally include a selectable marker, such as the neomycin resistance gene. In an embodiment, the expression cassette can be introduced into a cell using a viral vector.

In another aspect, a method for making a large fowl is provided. The method includes introducing a mutant $p27^{Kip1}$ gene lacking a Cdk2 phosphorylation site into the genome of a fowl cell by contacting *in vivo* a blastodermal cell of a fertilized cell with the mutant $p27^{Kip1}$ gene, wherein the $p27^{Kip1}$ gene is introduced directly into the germinal disk of the egg. Suitable fowl cells include those from chickens, ostriches, emus, turkeys, ducks, geese, quail, parrots, parakeets, cockatoos or cockatiels.

A further understanding of the present invention will be obtained by reference to the following description that sets forth illustrative embodiments.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

The present invention relates to nucleic acids encoding a mutant $p27^{Kip1}$ protein that lacks a Cdk2 phosphorylation site and to cells harboring mutant $p27^{Kip1}$ genes. In related

aspects, transgenic cells and transgenic animals are provided that have one or more mutant p27^Kip1 genes encoding protein that lacks a Cdk2 phosphorylation site.

The p27^Kip1 protein is phosphorylated by a Cdk at a phosphorylation site to create a recognition sequence for a SCF (*e.g.*, Cdk2). The absence or alteration of the Cdk2

5   phosphorylation site in p27^Kip1 reduces or eliminates phosphorylation. Although the mutant p27^Kip1 polypeptide is degraded in mid-G1 by the same pathway that degrades wildtype p27, the mutant p27^Kip1 polypeptide has a longer half life in the S phase of the cell cycle as compared with wildtype p27^Kip1 polypeptide. Yet, the mutant p27^Kip1 protein can retain other functions, such as the ability to inhibit Cdk2 *in vitro* kinase activity.

10   In one aspect of the invention, isolated mutant p27^Kip1 genes are provided for introduction into animal cells. (The term "isolated" refers to a molecule, such as a nucleic acid, or cell, that has been removed from its natural cellular environment. For example, an isolated nucleic acid is typically at least partially purified from other cellular nucleic acids, polypeptides and other constituents.) The mutant p27^Kip1 gene encodes a p27^Kip1 polypeptide

15   lacking a Cdk2 phosphorylation site, such that less than about 10% of the mutant p27^Kip1 polypeptide is phosphorylated at the Cdk2 phosphorylation site. In certain embodiments, phosphorylation at the Cdk2 phosphorylation site is less than about 5%, or less than about 1%.

The Cdk2 phosphorylation site can be defined by the following four amino acid

20   consensus sequence: (Ser/Thr)ProXaa(Lys/Arg) or the consensus sequence (Ser/Thr)ProXaa(Lys/Arg/His/Pro), wherein Xaa can be any amino acid residue. (*See, e.g.*, Holmes and Solomon, *J. Biol. Chem.* 271:25240-46 (1996).) Phosphorylation can be inhibited by substitutions, insertions and/or deletions (*e.g.*, 1-3 amino acid insertions or deletions).

25   Referring to Table 1, the Cdk2 phosphorylation site, including the phosphorylated residue, is generally conserved in p27^Kip1 polypeptides. As shown in the table, an asterisk indicates the position of a conserved threonine at position 187 of the human Cdk2 phosphorylation site. As used herein, this conserved threonine is referred to as threonine 187 (T187), although the skilled artisan will appreciate that this conserved residue may not

30   be at position 187 in all p27^Kip1 polypeptides. For example, in the mouse, hamster and rat polypeptide sequences, the conserved, phosphorylated residue is at position 186, although it is identifiable by sequence alignment and by biochemical analysis, as discussed in the Examples (*infra*). Thus, the terms "T187," "T187A" and position "187" are merely

Table 1

Consensus   151 IRKRPATDDSSTQNKRANRTEENVSDGSPNAGSVEQTPKKPGLRRRQT 198 (SEQ ID NO:4)
                                              *
                                          Residues

GenBank	Species				
7769665	Human	151	........L...	198	SEQ ID NO:5
4757962	Human	151	....	198	SEQ ID NO:6
12805035	Human	151	....	198	SEQ ID NO:6
2135228	Human	151	....	198	SEQ ID NO:6
3913222	Cat	151	..........P.......................................	198	SEQ ID NO:7
13429931	Pig	151	..........P..................SA...................	198	SEQ ID NO:8
6753386	Mouse	151	M.....AE....S...............T.....................	196	SEQ ID NO:9
2493565	Hamster	151	M.....A....S..............L.............H.........	198	SEQ ID NO:10
2102649	Rat	151	M.....AE....S...............T...............Q....	196	SEQ ID NO:11
2281010	Rat	151	M.....AE....S....S..........T...............Q....	196	SEQ ID NO:12

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shorthand for this conserved threonine residue position and not to be limited to amino acid 187 of a p27^{Kip1} polypeptide, or the corresponding codon in a p27^{Kip1} gene.

A Cdk2 phosphorylation site in a p27^{Kip1} polypeptide can be identified, for example, by biochemical analysis. (*See, e.g.,* Holmes and Solomon, *supra.*) A Cdk2 phosphorylation

5   site in a p27^{Kip1} gene and/or polypeptide sequence also can be identified by alignment with known p27^{Kip1} gene and/or polypeptide sequences. For example, an alignment can be performed by the local homology algorithm of Smith and Waterman (*Adv. Appl. Math.* 2:482 (1981), which is incorporated by reference herein in its entirety), by the homology alignment algorithm of Needleman and Wunsch (*J. Mol. Biol.* 48:443-53 (1970), which is incorporated

10   by reference herein in its entirety), by the search for similarity method of Pearson and Lipman (*Proc. Natl. Acad. Sci. USA* 85:2444-48 (1988), which is incorporated by reference herein in its entirety), by computerized implementations of these algorithms (*e.g.,* GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package (Accelrys), or by visual inspection.

15   One example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments to show the percent sequence identity. It also plots a tree or dendogram showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng and Doolittle (*J. Mol. Evol.* 25:351-60 (1987), which is

20   incorporated by reference herein in its entirety). The method used is similar to the method described by Higgins and Sharp (*Comput. Appl. Biosci.* 5:151-53 (1989), which is incorporated by reference herein in its entirety). The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids. The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences, producing

25   a cluster of two aligned sequences. This cluster is then aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences are aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise alignments. The program is run by designating specific sequences and their amino acid or nucleotide coordinates for regions of sequence

30   comparison and by designating the program parameters. For example, a reference sequence can be compared to other test sequences to determine the percent sequence identity relationship using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps.

Another example of an algorithm that is suitable for aligning sequences, and for determining percent sequence identity and sequence similarity, is the BLAST algorithm, which is described by Altschul et al. (J. Mol. Biol. 215:403-410 (1990), which is incorporated by reference herein in its entirety). (See also Zhang et al., Nucleic Acid Res.

5   26:3986-90 (1998); Altschul et al., Nucleic Acid Res. 25:3389-402 (1997), which are incorporated by reference herein in their entirety). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which

10   either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al., supra). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be

15   increased. Extension of the word hits in each direction is halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLAST

20   program uses as default parameters a wordlength (W) of 11, the BLOSUM62 scoring matrix (see Henikoff and Henikoff, Proc. Natl. Acad. Sci. USA 89:10915-19 (1992), which is incorporated by reference herein in its entirety) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands. The skilled artisan will appreciate, however, that other parameters can be used.

25   The isolated, mutant $p27^{Kip1}$ genes can be, for example, genomic DNA, cDNA, RNA, mRNA, and the like, as well as fragments of any of these. The mutant $p27^{Kip1}$ genes can be polynucleotides or nucleic acids or other polymers composed of a multiplicity of nucleotide units (ribonucleotide or deoxyribonucleotide or related structural variants) linked via phosphodiester bonds. Mutant genes can be of substantially any length, typically from about

30   twelve (12) nucleotides to about 10^9 nucleotides or larger, that do not encode a Cdk2 phosphorylation site. In one embodiment, a fragment of a mutant $p27^{Kip1}$ gene has at least 50 contiguous nucleotides; in other embodiments, the fragment of the mutant $p27^{Kip1}$ gene is at least 100 nucleotides, at least 200 nucleotides, at least 500 nucleotides, at least 1000 nucleotides, or more of the gene. In related embodiments, the mutant $p27^{Kip1}$ gene is at least

an exon, a cDNA, or a full length genomic p27^{Kip1} gene, lacking a Cdk2 phosphorylation site.

Mutant p27^{Kip1} genes also include derivatives, such as those based on all possible codon choices for an amino acid(s) that, when expressed from a mutant p27^{Kip1} gene, results
5   in the expression of a mutant protein in which Cdk-mediated phosphorylation is inhibited. At amino acid positions outside the Cdk2 phosphorylation site, mutant p27^{Kip1} gene derivatives can include those based on all possible codon choices for the same amino acid and codon choices based on conservative amino acid substitutions. For example, the following six groups each contain amino acids that are conservative substitutions for one
10   another: 1) Alanine (A), Serine (S), Threonine (T); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W). (*See also* Creighton, *Proteins*, W. H. Freeman and Company (1984).) In addition, individual substitutions, deletions or additions that alter, add or delete a single amino acid or a small
15   percentage of amino acids in an encoded sequence are also "conservative substitutions."

In certain embodiments, mutant p27^{Kip1} genes be synthesized, or chemically or biochemically modified (*e.g.*, can contain non-natural or derivatized nucleotide bases). Such modifications include, for example, labels, methylation, substitutions of one or more of the naturally-occurring nucleotides with an analog, internucleotide modifications such as
20   uncharged linkages (*e.g.*, methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, and the like), charged linkages (*e.g.*, phosphorothioates, phosphorodithioates, and the like), pendent moieties (*e.g.*, polypeptides), intercalators (*e.g.*, acridine, psoralen, and the like), chelators, alkylators, and modified linkages (*e.g.*, alpha anomeric nucleic acids, and the like).

25   The mutant p27^{Kip1} gene(s) can be homologous or heterologous to the cell or the animal. As used herein, the term "homologous" p27^{Kip1} gene refers to a p27^{Kip1} gene derived from the same species as the cell or animal. A "heterologous" p27^{Kip1} gene refers to a p27^{Kip1} gene from a different species. For example, if the animal is a chicken, a homologous mutant p27^{Kip1} gene is derived from a chicken p27^{Kip1} gene, while a heterologous mutant
30   p27^{Kip1} gene is derived, for example, from a mouse p27^{Kip1} gene.

The mutant p27^{Kip1} gene can be prepared by, for example, mutagenizing a wild-type p27^{Kip1} gene at one or more positions in the Cdk2 phosphorylation site. In various embodiments, the p27^{Kip1} gene is human, primate, mammalian, avian, porcine, ovine,

bovine, fowl, rodent, fish, crustacean, and the like. In specific embodiments, the p27^{Kip1} is from a sheep, goat, horse, cow, bull, pig, rabbit, guinea pig, hamster, rat, gerbil, mouse, chicken, ostrich, emu, turkey, duck, goose, quail, parrot, parakeet, cockatoo, cockatiel, trout, cod, salmon, crab, king crab, lobster, shrimp, and the like. p27^{Kip1} gene sequences are

5　　disclosed, for example, in the GenBank database under accession numbers gi|7769665|, gi|4757962|, gi|12805035|, gi|2135228|, gi|3913222|, gi|13429931|, gi|6753386|, gi|2493565|, gi|2102649|, and gi|2281010|, which are incorporated by reference herein in their entirety. p27^{Kip1} polypeptide sequences are disclosed, for example, in the GenBank database under accession numbers AAF69497.1, NP_004055.1, AAH01971.1, I52718, O19001,

10　　BAB39725.1, NP_034005.1, Q60439, BAA19960.1, and BAA21561.1 (the disclosures of which are incorporated by reference herein in their entirety).

　　　　p27^{Kip1} genes can be readily isolated by methods known to the skilled artisan. (*See generally* Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual*, 3rd ed., Cold Spring Harbor Publish., Cold Spring Harbor, NY (2001); Ausubel *et al.*, *Current Protocols in*

15　　*Molecular Biology*, 4th ed., John Wiley and Sons, New York (1999); which are incorporated by reference herein in their entirety.) Specific embodiments for the isolation of p27^{Kip1} genes, presented as example but not by way of limitation, are described below.

　　　　p27^{Kip1} genes can be isolated, for example, by polymerase chain reaction (PCR) to amplify the p27^{Kip1} gene, or a portion thereof, from a genomic or cDNA library.

20　　Oligonucleotide primers representing known p27^{Kip1} sequences can be used as primers in PCR. In a typical embodiment, the oligonucleotide primers represent at least a fragment of conserved segments of identity between p27^{Kip1} genes of different species. Synthetic oligonucleotides can be utilized as primers to amplify particular oligonucleotides within a p27^{Kip1} gene by PCR sequences from any suitable source (*e.g.*, RNA or DNA), typically a

25　　cDNA library or mRNA of potential interest. PCR can be carried out, for example, by use of a Perkin-Elmer Cetus thermal cycler and Taq polymerase (Gene Amp). Degenerate primers can be designed for use in the PCR reactions. For example, the CODEHOP strategy of Rose *et al.* (*Nucl. Acids Res.* 26:1628-35 (1998), which is incorporated by reference herein in its entirety) can be used to design degenerate PCR primers using multiply-aligned sequences as

30　　a reference. Methods for performing PCR and related methods are well known in the art. (*See, e.g.*, U.S. Patent Nos. 4,683,202; 4,683,195 and 4,800,159; Innis *et al.*, *PCR Protocols: A Guide to Methods and Applications*, Academic Press, Inc., San Diego, CA (1989); Innis *et al.*, *PCR Applications: Protocols for Functional Genomics*, Academic Press, Inc., San Diego, CA (1999); White (ed.), *PCR Cloning Protocols: From Molecular Cloning to*

Genetic Engineering, Humana Press, (1996); EP 320 308; the disclosures of which are incorporated by reference herein in their entirety.)

In an embodiment, degenerate primers are used to isolate the p27^{Kip1} cDNA from an avian species. Avian species are known to have p27^{Kip1}. (*See* Torchinsky *et al.*, *J.*
5   *Neurocytol.* 28:913-24 (1999).) Briefly, an alignment of multiple p27^{Kip1} polypeptide sequences from different animals is prepared and used to visually identify blocks of sequences having low codon degeneracy (*see* Rose *et al.* (*supra*)). The CODEHOP strategy of Rose *et al.* (*supra*) is used to design degenerate primers based on the blocks of low codon degeneracy. Pools of primers varying in redundancy from 2 fold to about 32 fold are
10   prepared. A hemi-nested PCR strategy is used to amplify fragments from an avian chicken cDNA library (*e.g.*, a chicken or hyacinth macaw library from Stratagene). Briefly, PCR is performed at 55°C using the primer pools. (*See, e.g.*, Rose *et al.* (*supra*); Rose *et al.*, *J. Virology* 71:4138-44 (1997).) PCR amplification products can be detected, for example, by agarose gel electrophoresis. The identity of the PCR amplification products can be
15   confirmed by DNA sequence analysis. Once the identify of the PCR amplification products is confirmed, the amplification products can be used to isolate full length p27^{Kip1} cDNA from the avian cDNA library. (*See, e.g.*, Sambrook *et al.*, *supra*; Ausubel *et al.*, *supra*.)

For expression cloning (a technique commonly known in the art), an expression library is constructed by methods known in the art. For example, mRNA is isolated, cDNA
20   is prepared and then ligated into an expression vector (*e.g.*, a bacteriophage derivative) such that it is capable of being expressed by the host cell into which it is then introduced. Various screening assays can then be used to select for the expressed p27^{Kip1} polypeptide. In one embodiment, polyclonal antibodies against a mammalian p27^{Kip1} polypeptide (*see, e.g.*, U.S. Patent No. 6,242,575; the disclosure of which is incorporated by reference herein in its
25   entirety) are used to screen a chicken cDNA expression library (*e.g.*, from Strategene) to identify avian p27^{Kip1} genes.

Alternatively, p27^{Kip1} genes can be isolated by hybridization using a heterologous p27^{Kip1} nucleic acid as a probe. For example, p27^{Kip1} genes can be isolated by screening a genomic or cDNA library with a p27^{Kip1} nucleic acid probe. Such a probe can be, for
30   example, a portion of a p27^{Kip1} gene or its specific RNA, or a fragment thereof, that exhibits low codon degeneracy. Such a probe can be prepared, detectably labeled, and used to screen a library by nucleic acid hybridization (*see, e.g.*, Benton and Davis, *Science* 196:180-82 (1977); Grunstein and Hogness, *Proc. Natl. Acad. Sci. USA* 72:3961-65 (1975); Sambrook *et*

al., supra; Ausubel *et al., supra*). DNA fragments with substantial identity to the probe will hybridize and can be identified using the detectable label.

In various embodiments, hybridization screening using a heterologous p27[Kip1] nucleic acid probe can assist in the isolation of p27[Kip1] genes. p27[Kip1] genes can be isolated, for example, from human or non-human sources, such as, for example, primate, porcine, bovine, feline, equine, canine, ovine, avian, reptilian, amphibian, piscine, and the like; and from non-vertebrate sources, such as insects, worms, nematodes, and the like. In certain embodiments, the isolated p27[Kip1] gene can be from a chicken, goose, duck, lobster, rabbit, sheep, cow, bull, horse, pig, and the like.

By way of example, and not limitation, procedures using low stringency conditions are as follows: Filters containing DNA are pretreated for 6 hours at 40°C in a solution containing 35% formamide, 5x SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% polyvinylpyrrolidone (PVP), 0.1% Ficoll, 1% bovine serum albumin (BSA), and 500 μg/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution with the following modifications: 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 μg/ml salmon sperm DNA, 10% (wt/vol) dextran sulfate, and 5-20 x 10^6 cpm ^{32}P-labeled probe. Filters are incubated in hybridization mixture for 18-20 hours at 40°C, and then washed for 1.5 hours at 55°C in a solution containing 2x SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.1% SDS. The wash solution is replaced with fresh solution and incubated an additional 1.5 hours at 60°C. Filters are blotted dry and exposed for autoradiography. If necessary, filters are washed for a third time at 65-68°C and re-exposed to film. Other conditions of low stringency that can be used are well known in the art (*e.g.*, those employed for cross-species hybridizations). (*See also* Shilo and Weinberg, *Proc. Natl. Acad. Sci. USA* 78:6789-92 (1981); Sambrook *et al., supra*; Ausubel *et al., supra*.)

Alternatively, moderate stringency conditions can be used. By way of example, and not limitation, procedures using such conditions of moderate stringency are as follows: Prehybridization of filters containing DNA is carried out for 8 hours to overnight at 55°C in buffer composed of 6x SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.2% Ficoll, 0.02% BSA and 500 μg/ml denatured salmon sperm DNA. Filters are hybridized for 24 hours at 55°C in a prehybridization mixture containing 100 μg/ml denatured salmon sperm DNA and 5-20 x 10^6 cpm of ^{32}P-labeled probe. Washing of filters is done at 37°C for 1 hour in a solution containing 2x SSC, 0.01% PVP, 0.01% Ficoll, and 0.01% BSA.

By way of example, and not limitation, procedures using conditions of high stringency are as follows: Prehybridization of filters containing DNA is carried out for 8 hours to overnight at 65°C in buffer composed of 6x SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 µg/ml denatured salmon sperm

5   DNA. Filters are hybridized for 48 hours at 65°C in prehybridization mixture containing 100 µg/ml denatured salmon sperm DNA and 5-20 x 10^6 cpm of ^{32}P-labeled probe. Washing of filters can be performed at 65°C for 1 hour in a solution containing 2x SSC, 0.01% PVP, 0.01% Ficoll, and 0.01% BSA. This is followed by a wash in 0.1x SSC at 50°C for 45 minutes before autoradiography. Other conditions of high stringency which can be used are

10   well known in the art. (See, e.g., Ausubel et al., supra; Sambrook et al., supra.)

Various other hybridization conditions can be used. For example, hybridization in 6x SSC at about 45°C, followed by washing in 2x SSC at 50°C can be used. Alternatively, the salt concentration in the wash step can range from low stringency of about 5x SSC at 50°C, to moderate stringency of about 2x SSC at 50°C, to high stringency of about 0.2x SSC at

15   50°C. In addition, the temperature of the wash step can be increased from low stringency conditions at room temperature, to moderately stringent conditions at about 42°C, to high stringency conditions at about 65°C. Other conditions include, but are not limited to, hybridizing at 68°C in 0.5M NaH_2PO_4 (pH7.2)/1 mM EDTA/7% SDS, or hybridization in 50% formamide/0.25M NaH_2PO_4 (pH 7.2)/0.25 M NaCl/1 mM EDTA/7% SDS, followed

20   by washing in 40 mM NaH_2PO_4 (pH 7.2)/1 mM EDTA/5% SDS at 50°C or in 40 mM NaH_2PO_4 (pH7.2)/ 1 mM EDTA/1% SDS at 50°C. Both temperature and salt can be varied, or alternatively, one or the other variable can remain constant while the other is changed.

Low, moderate and high stringency conditions are well known to those of skill in the art, and will vary predictably depending on the base composition of the particular nucleic

25   acid sequence and on the specific organism from which the nucleic acid sequence is derived. For guidance regarding such conditions see, for example, Sambrook et al. (supra) and Ausubel et al. (supra).

p27^{Kip1} genes can also be identified, for example, by searching a genomic sequence database, such as those for Drosophila, C. elegans, and the like. Such searches can be

30   performed, for example, using the Blast search engine (Altschul et al., Nucleic Acids Res. 25:3389-402 (1997)), or other suitable sequence comparison program. Information and tools for screening genomic databases are provided, for example, at the NCBI Internet web site (http://www.ncbi.nlm.nih.gov), as well as from commercially available sources. The

UniGene collection provides a non-redundant set of sequences that represent unique genes of different sequences. (*See* www.ncbi.nlm.nih.gov.) This collection includes well-characterized genes, as well as thousands of expressed sequence tag (EST) sequences.

The methods discussed above are not meant to limit the methods by which p27[Kip1]

5   genes can be isolated. p27[Kip1] genes derived from genomic DNA can contain regulatory and intron DNA regions in addition to coding regions; clones derived from cDNA will typically contain only exon sequences. Nucleic acids can be molecularly cloned into a suitable vector for propagation of those nucleic acids. (*See, e.g.*, Sambrook *et al., supra;* Ausubel *et al., supra.*)

10   A p27[Kip1] gene can be mutagenized to create a substitution, deletion and/or insertion in the Cdk2 phosphorylation site. In an exemplary embodiment, a substitution of the phosphorylated threonine or serine is made by altering the codon that codes for that residue. In other embodiments, other residues in the Cdk2 phosphorylation site can be changed or deleted. This can be accomplished, for example, by site-directed mutagenesis using the

15   Amersham technique (Amersham mutagenesis kit, Amersham, Inc., Cleveland, Ohio) based on the methods of Taylor *et al.* (*Nucl. Acids Res.* 13:8749-84 (1985); *Nucl. Acids Res.* 13:8764-85 (1985)), Nakamaye and Eckstein (*Nucl. Acids Res.* 14:9679-98 (1986)); and Dente *et al.* (*DNA Cloning*, Glover, Ed., IRL Press, pp. 791-802 (1985)); using a Promega kit (Promega Inc., Madison, Wis.); using a Biorad kit (Biorad Inc., Richmond, Calif.), based

20   on the methods of Kunkel (*Proc. Natl. Acad. Sci. USA* 82:488-92 (1985); *Meth. Enzymol.* 154:367-82 (1987); U.S. Patent No. 4,873,192), and the like. Site directed mutagenesis can also be accomplished using PCR-based mutagenesis, such as the technique described by Zhengbin *et al.* (in *PCR Methods and Applications*, Cold Spring Harbor Laboratory Press, New York, pp. 205-207 (1992)), by Jones and Howard (*BioTechniques* 8:178-83 (1990);

25   *BioTechniques* 10:62-66 (1991)); by Ho *et al.* (*Gene* 77:51-59 (1989)), and by Horton *et al.* (*BioTechniques* 8:528-35 (1990); *Gene* 77:61-68 (1989)). Other methods of mutagenizing a p27[Kip1] gene to modify a Cdk2 phosphorylation site are known to the skilled artisan and are within the scope of the invention.

A mutant p27[Kip1] gene can be part of an expression cassette, *i.e.*, having a promoter

30   and a coding region encoding a mutant p27[Kip1] polypeptide. The promoter can be a homologous promoter (*i.e.*, a p27[Kip1] gene promoter from the same species) or a heterologous promoter (*i.e.*, a p27[Kip1] gene promoter from a different species, or a non-p27[Kip1] gene promoter) for expression of a mutant p27[Kip1] coding region (*i.e.*, lacking a Cdk2 phosphorylation site). As used herein, the term "coding region" refers to a nucleotide

sequence containing a translational initiation codon followed by an ordered arrangement of codons that encode a mutant p27^{Kip1} protein and a translational termination codon. A "coding region" can also encode a fragment of a mutant p27^{Kip1} protein lacking a Cdk2 phosphorylation site. The promoter is operably or operatively associated with the coding

5   region, whereby the promoter effects expression of the coding region.

Suitable heterologous promoters include, for example, promoters that are expressed in a wide variety of tissue types, such as, for example, the SV40 early promoter region (Benoist and Chambon, *Nature* 290:304-10 (1981)), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto *et al.*, *Cell* 22:787-97 (1980)), the herpes

10   thymidine kinase promoter (Wagner *et al.*, *Proc. Natl. Acad. Sci. USA* 78:1441-45 (1981)), the regulatory sequences of the metallothionein gene (Brinster *et al.*, *Nature* 296:39-42 (1982)), the cytomegalovirus (CMV) promoter, the mouse Oct4 gene promoter (International Patent Publication No. WO 00/56932), the Mouse Moloney Leukemia Virus LTR (Miller and Buttimore, *Mol. Cell. Biol.* 6:2895-902 (1986), Gossen and Bujard, *Proc. Natl. Acad.*

15   *Sci. USA* 89:5547-51 (1992); Pescini *et al.*, *Biochem. Biophys Res. Comm.* 202:1664-67 (1994); ubiquitously expressed promoters such as the ROSA26 and G3BP promoters (Zambrowicz *et al.*, *Proc. Natl. Acad. Sci. USA* 94:3789-94 (1997); Parker *et al.*, *Molecular and Cellular Biology* 16:2561-69 (1996)); and the like.

For expression in avian species, the promoter can be, for example, lactoferrin-derived

20   transcription regulatory sequences (International Publication No. WO 00/75300), the chicken ovalbumin promoter (Genbank Accession Nos. J00895 or M24999), the chicken lysozyme promoter (Genbank Accession Nos. J00886 or V00429), and the like. Other suitable promoters are known to those of skill in the art. In certain embodiments, an Internal Ribosomal Entry Site (IRES) can be part of a promoter system express a mutant p27^{Kip1}

25   gene. Suitable polyadenylation sequences include, for example, the human beta-globin polyadenylation sequence, and the SV40 early polyadenylation sequence.

The mutant p27^{Kip1} gene expression cassette optionally can further include a selectable marker, such as a positively and/or negatively selectable marker. Suitable positively selectable markers can include, for example, the neomycin gene, the hygromycin

30   gene, the *hisD* gene, the xanthine-guanine phosphoribosyltransferase (Gpt) gene conferring resistance to mycophenolic acid (Mulligan *et al.*, *Proc. Natl. Acad. Sci. USA* 78:2072-76 (1981)), the hypoxanthine phosphoribosyl transferase (Hprt) gene, and the like. Suitable negative selection markers include, for example, the HSV thymidine kinase gene, the Hprt gene, the Gpt gene, Diphtheria toxin, Ricin toxin, cytosine deaminase, and the like. The

selectable marker typically confers a phenotype for identification and isolation of cells containing an introduced mutant p27^{Kip1} gene.

A mutant p27^{Kip1} gene optionally can be part of an expression vector. Such an

5   expression vector typically comprises an expression cassette (*e.g.*, a promoter operably linked to a mutant p27^{Kip1} gene operably linked to a polyadenylation sequence), one or more origins of replication, and, optionally, one or more selectable markers (*e.g.*, an antibiotic resistance gene and/or any of those describe above). Suitable origins of replication include, for example, the SV40 origin of replication, the colE1 origin of replication, and the like.

Suitable expression vectors can include defective or attenuated retroviral vectors or

10   other viral vector (*see, e.g.*, U.S. Patent No. 4,980,286). For example, a retroviral vector, as described by Miller *et al.* (*Meth. Enzymol.* 217:581-99 (1993)) can be used. (*See also* Boesen *et al.*, *Biotherapy* 6:291-302 (1994).) (These references are incorporated herein in their entirety.) These retroviral vectors are typically modified to delete retroviral sequences that are not necessary for packaging of the viral genome and integration into host cell DNA.

15   The mutant p27^{Kip1} gene is inserted into the vector, which facilitates delivery of the gene into a cell. Lentiviral vectors can also be used. (*See, e.g.*, Naldini *et al.*, *Science* 272:263-67 (1996), incorporated by reference herein in its entirety.)

Adenoviruses can also be used as an expression vector to introduce a mutant p27^{Kip1} gene into cells. Adenoviruses have the advantage of being capable of infecting non-dividing

20   cells. Adeno-associated virus (AAV) are another suitable vector. (*See, e.g.*, Ali *et al.*, *Gene Therapy* 1:367-84 (1994); U.S. Patent Nos. 4,797,368 and 5,139,941; Walsh *et al.*, *Proc. Soc. Exp. Biol. Med.* 204:289-300 (1993); Grimm *et al.*, *Human Gene Therapy* 10:2445-50 (1999); the disclosures of which are incorporated by reference herein in their entirety.)

The expression cassette or vector can be used for homologous integration of a mutant

25   p27^{Kip1} gene at a predetermined locus in the genome of a cell. For example, a mutant p27^{Kip1} gene can be homologously integrated at an endogenous p27^{Kip1} locus in a cell. Alternatively, a mutant p27^{Kip1} gene can be integrated at any other suitable locus in a cell, such as a non-essential gene locus or other non-essential genomic region. As used herein, the term "homologous recombination" refers to a process of recombination or gene conversion

30   whereby homology regions flanking a mutant p27^{Kip1} gene, or a portion thereof (*e.g.*, the nucleic acid sequence encoding a Cdk2 phosphorylation site), replace corresponding chromosomal sequences in the genome of the cell.

Homologous recombination can occur by, for example, double-crossover replacement recombination, in which homologous recombination (*e.g.*, strand exchange, strand pairing, strand scission, and strand ligation) occurs between homology regions in an expression vector or expression construct and chromosomal sequences in a cell. The

5 homology regions are generally used in the same orientation (*e.g.*, the upstream direction (5' relative to the direction of transcription) is the same for each homology region) to avoid rearrangements. Double-crossover replacement recombination thus can be used to insert a mutant p27^{Kip1} gene, or a portion thereof, into an endogenous gene locus. In certain embodiments, the homology regions are from an endogenous p27^{Kip1} gene, and the mutant

10 p27^{Kip1} gene or a fragment thereof, integrates at an endogenous p27^{Kip1} gene locus. Alternatively, the homologous regions are from a different locus, and the mutant p27^{Kip1} gene is integrated at that locus.

Suitable "targeting constructs" for homologous integration of a mutant p27^{Kip1} gene include, for example, those disclosed in U.S. Patent Nos. 5,631,153; 5,627,059; 5,487,992;

15 5,464,764; and 6,204,061 (the disclosures of which are incorporated by reference herein in their entirety). Targeting constructs can be, for example, a targeting construct for single-crossover integration, or "hit-and-run" targeting, which has only a single homology region linked to a mutant p27^{Kip1} gene or gene fragment. Alternatively, the targeting construct can have two homology regions, each flanking a mutant p27^{Kip1} gene or gene fragment. For

20 example, a targeting construct can comprise, in order: (1) a first homology region having a sequence substantially identical to a sequence of a portion of an endogenous gene locus, (2) a mutant p27^{Kip1} gene or a fragment thereof, and (3) a second homology region having a sequence substantially identical to a different portion of the endogenous gene locus. In certain embodiments, the targeting construct further comprises a negatively selectable

25 marker (*e.g.*, Diphtheria toxin gene with the PGK promoter driving transcription) linked to an outer end of a homology region. Such a targeting construct can optionally further include a positively selectable marker disposed between the first and second homology regions. The homology regions typically range from between about 50 base pairs to about several tens of kilobases. In some embodiments, targeting constructs are generally at least about 250

30 nucleotides, at least about 500 nucleotides, typically at least about 1000 to about 6000 nucleotides, or longer.

The homology region(s) can be selected at the discretion of the practitioner on the basis of the sequence composition and complexity of the gene locus and guidance provided in the art (*see, e.g.*, Hasty *et al.*, *Mol. Cell. Biol.* 11:5586-91 (1991); Shulman *et al.*, *Mol.*

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Cell. Biol. 10:4466-72 (1990), which are incorporated herein by reference in their entirety).
Targeting constructs are generally double-stranded DNA molecules; most are typically
linear. General principles regarding the construction of targeting constructs and selection
methods are reviewed in Bradley *et al.* (*Bio/Technology* 10:534-39 (1992), incorporated
5   herein by reference in its entirety). (*See also* Capecchi, *Science* 244:1288-92 (1989);
incorporated herein by reference in its entirety.)

        In another aspect, transgenic cells comprising one or more mutant p27^{Kip1} genes are
provided. As used herein, the term "transgenic cells" refers to a human or non-human cell
comprising one or more mutant p27^{Kip1} genes. A transgenic cells can be, for example, from
10   a human, primate, mammal, avian, porcine, ovine, bovine, feline, canine, fowl, rodent, fish,
crustacean, and the like. In specific embodiments, the transgenic cells can be from a sheep,
goat, horse, cow, bull, pig, rabbit, guinea pig, hamster, rat, gerbil, mouse, chicken, ostrich,
emu, turkey, duck, goose, quail, parrot, parakeet, cockatoo, cockatiel, trout, cod, salmon,
crab, king crab, lobster, or shrimp.

15   Mutant p^{27Kip1} genes can be introduced into target cells, such as, for example,
pluripotent or totipotent cells such as embryonic stem (ES) cells (*e.g.*, murine embryonal
stem cells or human embryonic stem cells) or other stem cells (*e.g.*, adult stem cells); germ
cells (*e.g.*, primordial germ cells, oocytes, eggs, spermatocytes, or sperm cells); fertilized
eggs; fetal or adult somatic cells, either differentiated or undifferentiated (*e.g.*, thymocytes,
20   fibroblasts, keratinocytes, brain, muscle, liver, lung, bone marrow, heart, neuron,
gastrointestinal, kidney, spleen, or epithelial cells); and the like. In certain embodiments, the
mutant p27Kip1 gene can be introduced into embryonic stem cells or germ cells.

        Suitable transgenic cells can also include "cell lines," which refers to individual cells,
harvested cells, and cultures containing the cells derived from cells of the cell line referred
25   to. A cell line is said to be "continuous," "immortal," or "stable" if the line remains viable
over a prolonged time, typically at least about six months. Suitable transgenic cells can also
include primary cells. Primary cells include cells that are obtained directly from an
organism or that are present within an organism, and cells that are obtained from these
sources and grown in culture, but are not capable of continuous (*e.g.*, many generations)
30   growth in culture. For example, primary fibroblast cells are considered primary cells. Cells
can be modified *in vitro*, *ex vivo*, or *in vivo*.

        In a related aspect, transgenic animals harboring one or more mutant p27^{Kip1} genes,
and methods of making such animals, are provided. As used herein, the term "transgenic

animal" refers to a non-human animal that harbors cells containing one or more mutant p27^{Kip1} genes. A transgenic animal can be, for example, a primate, mammal, avian, porcine, ovine, bovine, feline, canine, fowl, rodent, fish, crustacean, and the like. In specific embodiments, the transgenic animal can be a sheep, goat, horse, cow, bull, pig, rabbit,

5   guinea pig, hamster, rat, gerbil, mouse, chicken, ostrich, emu, turkey, duck, goose, quail, parrot, parakeet, cockatoo, cockatiel, trout, cod, salmon, crab, king crab, lobster, or shrimp. Transgenic animals include chimeric animals (*i.e.*, those composed of a mixture of genetically different cells), mosaic animals (*i.e.*, an animal composed of two or more cell lines of different genetic origin or chromosomal constitution, both cell lines derived from the

10   same zygote), immature animals, fetuses, blastulas, and the like.

        In mutant p27^{Kip1} transgenic animals, the mutant p27^{Kip1} gene causes an increased size of at least a portion of the animal, as compared with wild-type, non-transgenic animal (*i.e.*, not having a mutant p27^{Kip1} gene). In certain embodiments, the mutant p27^{Kip1} transgenic animals have enlarged tissues that contain more cells or larger cells than tissues from a non-transgenic animal. In other embodiments, mutant p27^{Kip1} animals exhibit

15   increased female fertility, reduced pituitary tumorigenesis, and reduced retinal architecture disruption, as compared with animals having p27^{Kip1} gene disruption(s) or knockout(s) (*i.e.*, loss of p27^{Kip1} function). Transgenic animals can contain one or more mutant p27^{Kip1} genes at the endogenous p27^{Kip1} locus, and/or at a non-p27^{Kip1} locus (or loci). The transgenic

20   animals can be homozygous or heterozygous for the mutant p27^{Kip1} gene.

        Transgenic, non-human animals containing a mutant p27^{Kip1} gene can be prepared by methods known in the art. In general, a mutant p27^{Kip1} gene is introduced into target cells, which are then used to prepare a transgenic animal. Mutant p27^{Kip1} genes can be introduced into target cells, such as for example, pluripotent or totipotent cells such as embryonic stem

25   (ES) cells (*e.g.*, murine embryonal stem cells or human embryonic stem cells) or other stem cells (*e.g.*, adult stem cells); germ cells (*e.g.*, primordial germ cells, oocytes, eggs, spermatocytes, or sperm cells); fertilized eggs; zygotes; blastomeres; and the like; fetal or adult somatic cells (either differentiated or undifferentiated); and the like. In certain embodiments, the mutant p27^{Kip1} gene can be introduced into embryonic stem cells or germ

30   cells of animals (*e.g.*, mammals, farm animals, livestock, hatchery animals, and the like) to prepare a mutant p27^{Kip1} transgenic animal.

        Embryonic stem cells can be manipulated according to published procedures (*see, e.g., Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, Robertson (ed.), IRL Press, Washington, D.C. (1987); Zjilstra *et al., Nature* 342:435-38 (1989);

Schwartzberg *et al.*, *Science* 246:799-803 (1989); U.S. Patent Nos. 6,194,635; 6,107,543;

and 5,994,619; each of which is incorporated herein by reference in their entirety). Methods

for isolating primordial germ cells are well known in the art. For example, methods of

isolating primordial germ cells from ungulates are disclosed in U.S. Patent No. 6,194,635

5   (the disclosure of which is incorporated by reference herein in its entirety). Briefly,

primordial germ cells are isolated from gonadal ridges of an embryo at a particular stage in

development (*e.g.*, day-25 porcine embryos or day 34-40 bovine embryos). The stage of

development at which primordial germ cells are extracted from an embryo of a particular

species will vary with the species, as will be appreciated by the skilled artisan.

10   Determination of the appropriate embryonic developmental stage for such extraction is

readily performed using the guidance provided herein and ordinary skill in the art.

       Primordial germ cells can be isolated from the dorsal mesentery and usually test

positive for alkaline phosphate activity. The cells can be isolated at a suitable time after

fertilization. To ascertain that harvested cells are of an appropriate developmental age,

15   harvested cells can be tested for morphological criteria which can be used to identify

primordial germ cells which are pluripotent (*see, e.g.*, DeFelici and McLaren, *Exp. Cell Res.*

142:476-82 (1982)). To further substantiate pluripotency, a sample of the extracted cells can

be subsequently tested for alkaline phosphatase (AP) activity. Pluripotent cells, such as

primordial germ cells, can share markers typically found on stem cells. Primordial or

20   embryonic germ cells typically manifest alkaline phosphatase (AP) activity, and AP positive

cells are typically germ cells. AP activity is rapidly lost with differentiation of embryonic

germ cells *in vitro*. Expression of AP also has been demonstrated in ES and ES-like cells in

the mouse (*see, e.g.*, Wobus *et al.*, *Exp. Cell. Res.* 152:212-19 (1984); Pease *et al.*, *Dev. Bio.*

141:344-52 (1990)), rat (*see, e.g.*, Ouhibi *et al.*, *Mol. Repro. Dev.* 40:311-24 (1995)), pig

25   (*see, e.g.*, Talbot *et al.*, *Mol. Repro. Dev.* 36:139-47 (1993)) and bovine animals (*see, e.g.*,

Talbot *et al.*, *Mol. Repro. Dev.* 42:35-52 (1995)). AP activity has also been detected in

murine primordial germ cell (*see, e.g.*, Chiquoine, *Anat. Rec.* 118:135-46 (1954)), murine

embryonic germ cells (*see, e.g.*, Matsui *et al.*, *Cell* 70:841-47 (1992); Resnick *et al.*, *Nature*

359:550-51 (1992)) and porcine primordial germ cells.

30   In a particular embodiment, transgenic avian animals can be prepared using avian

primordial germ cells. Such methods are disclosed, for example, in U.S. Patent No.

5,156,569 (the disclosure of which is incorporated by reference herein in its entirety).

Generally, primordial germ cells are isolated and cultured in the presence of growth factors,

such as, for example, leukemia inhibiting factor (LIF), stem cell factor (SCF), insulin-like growth factor (IGF) and/or basic fibroblast growth factor (bFGF).

Methods for isolation of primordial germ cells from donor avian embryos have been reported in the literature and can be effected by one skilled in the art. (*See, e.g.*, JP 924997

5   (Pub. No. 05-227947); Chang *et al., Cell Biol. Int.* 19:143-49 (1992); Naito *et al., Mol. Reprod. Devel.* 39:153-61 (1994); Yasuda *et al., J. Reprod. Fert.* 96:521-28 (1992); Chang *et al., Cell Biol. Int. Reporter* 16:853-57 (1992); each of which is incorporated by reference in their entirety therein.) In one example, primordial germ cells are isolated from chicken eggs which have been incubated for about 53 hours (stage 12-14 of embryonic

10   development), embryos are removed, embryonic cells are collected from the dorsal aorta thereof, and transferred to suitable cell culture medium (*e.g.*, M199 medium). These primordial germ cells can be purified (*e.g.*, by Ficoll density centrifugation) and resuspended in growth factor-containing culture medium. The isolated primordial germ cells are then counted and separated manually (*e.g.*, using a pipette). To increase the number of primordial

15   germ cells, cells can be collected from multiple avian embryos and pooled. The isolated primordial germ cells can be incubated in a suitable growth factor-containing medium. For example, one suitable culture medium includes α-MEM, containing 10% fetal calf serum, 2 mM L-glutamine, 0.56% antibiotic/antimitotic, 34.56 mM β-mercaptoethanol, 0.00625 U/μl of LIF, 0.25 pg/μl of bFGF, 0.5625 pg/μl of IGF and 4.0 pg/μl of SCF.

20   Mutant $p27^{Kip1}$ genes can be introduced into target cells by any suitable method. For example, a mutant $p27^{Kip1}$ gene(s) can be introduced into a cell by transfection (*e.g.*, calcium phosphate or DEAE-dextran mediated transfection), lipofection, electroporation, microinjection (*e.g.*, by direct injection of naked DNA), biolistics, infection with a viral vector containing a mutant $p27^{Kip1}$ gene, cell fusion, chromosome-mediated gene transfer,

25   microcell-mediated gene transfer, nuclear transfer, and the like.

In certain embodiments, a mutant $p27^{Kip1}$ gene is introduced into target cells by transfection or lipofection. Suitable agents for transfection or lipofection include, for example, calcium phosphate, DEAE dextran, lipofectin, lipfectamine, DIMRIE C, Superfect, and Effectin (Qiagen), unifectin, maxifectin, DOTMA, DOGS (Transfectam;

30   dioctadecylamidoglycylspermine), DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine), DOTAP (1,2-dioleoyl-3-trimethylammonium propane), DDAB (dimethyl dioctadecylammonium bromide), DHDEAB (N,N-di-n-hexadecyl-N,N-dihydroxyethyl ammonium bromide), HDEAB (N-n-hexadecyl-N,N-dihydroxyethylammonium bromide),

polybrene, poly(ethylenimine) (PEI), and the like. (*See, e.g.*, Banerjee *et al.*, *Med. Chem.* 42:4292-99 (1999); Godbey *et al.*, *Gene Ther.* 6:1380-88 (1999); Kichler *et al.*, *Gene Ther.* 5:855-60 (1998); Birchaa *et al.*, *J. Pharm.* 183:195-207 (1999); each incorporated by reference herein in its entirety.)

5   For avian species, which form a shell, the optimal time to introduce a mutant p27^{Kip1} gene, into avian cells is after oviposition and within six hours of activation (post-incubation) so that the cells have started to grow but have not undergone a cell division. Oviposition is the time at which the egg is laid. In the chicken, oviposition typically occurs at about 20 hours of uterine age. Mutant p27^{Kip1} genes can be introduced into the blastoderm or

10   germinal disc after oviposition, but before incubation of the egg (*i.e.*, before the first cell division after the egg is incubated). The germinal disc is distinguished from the germinal crescent region in that the germinal disc contains undifferentiated blastodermal cells, whereas the germinal crescent region appears in the early stages of chick embryo development.

15   In certain embodiments, the blastoderm is accessed by cutting or drilling a small hole in the egg shell (sitting upright) with a scalpel or drill and gently peeling back the inner membrane to expose the white albumen. The blastoderm orients to the top of the yolk and is visualized under light. The cells of the blastoderm can be transfected *in vivo* by infusing nucleic acids (*e.g.*, DNA) directly into the blastoderm using a syringe and small gauge

20   needle. The nucleic acid can be naked or complexed with lipids or other suitable compounds to facilitate DNA uptake (*e.g.*, DEAE-dextran). If the DNA is naked, the transfection efficiency can be increased by passing an electrical current across the blastoderm or whole egg with a device, such as a human heart defibrillator. If a current is passed across the whole egg, two additional holes are made in the egg shell to expose the inner membrane to the

25   current since the shell will not conduct electricity.

         Alternatively, the blastoderm can be removed from the egg and pooled with cells from several eggs (*e.g.*, using a small pipet). *In vitro*, nucleic acid uptake by blastodermal cells is facilitated by such techniques as electroporation, DEAE-dextran treatment, calcium phosphate treatment, lipofection, and the like. Following transfection, the cells can be

30   transferred into the germinal disc of an unfertilized egg for development of a transgenic chick.

         The overall efficiency of the nucleic acid delivery procedure to avian cells can depend on the methods and timing of gene delivery. Transfection efficiency is optionally

increased by, for example, subjecting the blastoderm or cells derived from the blastoderm to several rounds of transfection or adding a selectable marker to the mutant p27[Kip1] gene and infusing antibiotic, or other suitable drug, into the yolk or testes following transfection or cell transfer.

5   The mutant p27[Kip1] genes also can be introduced into cells by electroporation (*see*, *e.g.*, Wong and Neumann, *Biochem. Biophys. Res. Commun.* 107:584-87 (1982)) and biolistics (*e.g.*, a gene gun; Johnston and Tang, *Methods Cell Biol.* 43 Pt A:353-65 (1994); Fynan *et al.*, *Proc. Natl. Acad. Sci. USA* 90:11478-82 (1993)).

       Methods of introducing mutant p27[Kip1] genes into target cells further include
10   microinjection of the gene into target cells. For example, a mutant p27[Kip1] gene can be microinjected into pronuclei of fertilized oocytes or the nuclei of ES cells. A typical method is microinjection of the fertilized oocyte. The fertilized oocytes are microinjected with nucleic acids encoding mutant p27[Kip1] genes by standard techniques. The microinjected oocytes are typically cultured *in vitro* until a "pre-implantation embryo" is obtained. Such a
15   pre-implantation embryo typically contains approximately 16 to 150 cells. The 16 to 32 cell stage of an embryo is commonly referred to as a "morula." Those pre-implantation embryos containing more than 32 cells are commonly referred to as "blastocysts." They are generally characterized as demonstrating the development of a blastocoel cavity typically at the 64 cell stage. Methods for culturing fertilized oocytes to the pre-implantation stage include those
20   described by Gordon *et al.* (*Methods in Enzymology* 101:414 (1984)); Hogan *et al.* (in *Manipulating the Mouse Embryo*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1986)); Hammer *et al.* (*Nature* 315:680 (1986)); Gandolfi *et al.* (*J. Reprod. Fert.* 81:23-28 (1987)); Rexroad *et al.* (*J. Anim. Sci.* 66:947-53 (1988)); Eyestone *et al.* (*J. Reprod. Fert.* 85:715-20 (1989)); Camous *et al.* (*J. Reprod. Fert.* 72:779-85 (1989)); and
25   Heyman *et al.* (*Theriogenology* 27:5968 (1989)) for mice, rabbits, pigs, cows, and the like. (These references are incorporated herein in their entirety.) Such pre-implantation embryos can be thereafter transferred to an appropriate (*e.g.*, pseudopregnant) female by standard methods. Depending upon the stage of development when the mutant p27[Kip1] gene, or the mutant p27[Kip1] gene-containing cell is introduced into the embryo, a chimeric or mosaic
30   animal can result. As is well known, mosaic and chimeric animals can be bred to form true germline mutant p27[Kip1] transgenic animals by selective breeding methods well-known in the art. Alternatively, microinjected or transfected embryonic stem cells can be injected into appropriate blastocysts and then the blastocysts are implanted into the appropriate foster females (*e.g.*, pseudopregnant females).

A mutant p27^{Kip1} gene also can be introduced into cells by infection of cells or into cells of a zygote with an infectious virus containing the mutant gene. Suitable viruses include retroviruses (*see generally* Jaenisch, *Proc. Natl. Acad. Sci. USA* 73:1260-64 (1976)); defective or attenuated retroviral vectors (*see, e.g.*, U.S. Patent No. 4,980,286; Miller *et al.*,

5   *Meth. Enzymol.* 217:581-99 (1993); Boesen *et al.*, *Biotherapy* 6:291-302 (1994); these references are incorporated herein in their entirety), lentiviral vectors (*see, e.g.*, Naldini *et al.*, *Science* 272:263-67 (1996), incorporated by reference herein in its entirety), adenoviruses or adeno-associated virus (AAV) (*see, e.g.*, Ali *et al.*, *Gene Therapy* 1:367-84 (1994); U.S. Patent Nos. 4,797,368 and 5,139,941; Walsh *et al.*, *Proc. Soc. Exp. Biol. Med.*

10   204:289-300 (1993); Grimm *et al.*, *Human Gene Therapy* 10:2445-50 (1999); the disclosures of which are incorporated by reference herein in their entirety).

Viral vectors can be introduced into, for example, embryonic stem cells, primordial germ cells, oocytes, eggs, spermatocytes, sperm cells, fertilized eggs, zygotes, blastomeres, or any other suitable target cell. In an exemplary embodiment, retroviral vectors which

15   transduce dividing cells (*e.g.*, vectors derived from murine leukemia virus; *see, e.g.*, Miller and Baltimore, *Mol. Cell. Biol.* 6:2895 (1986)) can be used. The production of a recombinant retroviral vector carrying a gene of interest is typically achieved in two stages. First, a mutant p27^{Kip1} gene can be inserted into a retroviral vector which contains the sequences necessary for the efficient expression of the mutant p27^{Kip1} gene (including

20   promoter and/or enhancer elements which can be provided by the viral long terminal repeats (LTRs) or by an internal promoter/enhancer and relevant splicing signals), sequences required for the efficient packaging of the viral RNA into infectious virions (*e.g.*, a packaging signal (Psi), a tRNA primer binding site (-PBS), a 3' regulatory sequence required for reverse transcription (+PBS)), and a viral LTRs). The LTRs contain sequences required

25   for the association of viral genomic RNA, reverse transcriptase and integrase functions, and sequences involved in directing the expression of the genomic RNA to be packaged in viral particles.

Following the construction of the recombinant vector, the vector DNA is introduced into a packaging cell line. Packaging cell lines provide viral proteins required in *trans* for

30   the packaging of viral genomic RNA into viral particles having the desired host range (*i.e.*, the viral-encoded core (gag), polymerase (pol) and envelope (env) proteins). The host range is controlled, in part, by the type of envelope gene product expressed on the surface of the viral particle. Packaging cell lines can express ecotrophic, amphotropic or xenotropic envelope gene products. Alternatively, the packaging cell line can lack sequences encoding

a viral envelope (env) protein. In this case, the packaging cell line can package the viral genome into particles which lack a membrane-associated protein (*e.g.*, an env protein). To produce viral particles containing a membrane-associated protein which permit entry of the virus into a cell, the packaging cell line containing the retroviral sequences can be

5   transfected with sequences encoding a membrane-associated protein (*e.g.*, the G protein of vesicular stomatitis virus (VSV)). The transfected packaging cell can then produce viral particles which contain the membrane-associated protein expressed by the transfected packaging cell line; these viral particles which contain viral genomic RNA derived from one virus encapsidated by the envelope proteins of another virus are said to be pseudotyped virus

10   particles.

Oocytes which have not undergone the final stages of gametogenesis are typically infected with the retroviral vector. The injected oocytes are then permitted to complete maturation with the accompanying meiotic divisions. The breakdown of the nuclear envelope during meiosis permits the integration of the proviral form of the retrovirus vector

15   into the genome of the oocyte. When pre-maturation oocytes are used, the injected oocytes are then cultured *in vitro* under conditions that permit maturation of the oocyte prior to fertilization *in vitro*. Conditions for the maturation of oocytes from a number of mammalian species (*e.g.*, bovine, ovine, porcine, murine, and caprine) are well known in the art. In general, a base medium for *in vitro* maturation of bovine oocytes can be used (*e.g.*, TC-

20   M199 medium supplemented with hormones (*e.g.*, luteinizing hormone and estradiol)). Other media for the maturation of oocytes can be used for the *in vitro* maturation of other mammalian oocytes and are well known to the skilled artisan. The amount of time a pre-maturation oocyte is exposed to maturation medium to permit maturation varies between mammalian species, as is known to the skilled artisan. For example, an exposure of about 24

25   hours is sufficient to permit maturation of bovine oocytes, while porcine oocytes require about 44-48 hours.

Oocytes can be matured *in vivo* and employed in place of oocytes matured *in vitro*. For example, when porcine oocytes are employed, matured pre-fertilization oocytes can be harvested directly from pigs that are induced to superovulate. Briefly, on day 15 or 16 of

30   estrus, a female pig(s) can be injected with about 1000 units of pregnant mare's serum (PMS; available from Sigma and Calbiochem). Approximately 48 hours later, the pig(s) is injected with about 1000 units of human chorionic gonadotropin) (hCG; Sigma), and 24-48 hours later matured oocytes are collected from oviduct. These *in vivo* matured pre-fertilization oocytes can then be injected with the desired preparation. Methods for the

superovulation and collection of *in vivo* matured (*e.g.*, oocytes at the metaphase 2 stage) oocytes are known for a variety of mammals (*e.g.*, for superovulation of mice, *see* Hogan *et al.*, in *Manipulating the Mouse Embryo: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1994), pp. 130-133; the disclosure of which is

5   incorporated by reference herein in its entirety).

Retroviral vectors capable of infecting the desired species of non-human animal can be grown and concentrated to very high titers (*e.g.*, 1×10^8 cfu/ml). The use of high titer virus stocks allows the introduction of a defined number of viral particles into the perivitelline space of each injected oocyte. The perivitelline space of most mammalian

10   oocytes can accommodate about 10 picoliters of injected fluid (those skilled in the art know that the volume that can be injected into the perivitelline space of a mammalian oocyte or zygote varies somewhat between species as the volume of an oocyte is smaller than that of a zygote and thus, oocytes can accommodate somewhat less than can zygotes). The virus stock can be titered and diluted prior to microinjection into the perivitelline space so that the

15   number of proviruses integrated in the resulting transgenic animal is controlled. The use of pre-maturation oocytes or mature fertilized oocytes as the recipient of the virus minimizes the production of animals which are mosaic for the provirus as the virus integrates into the genome of the oocyte prior to the occurrence of cell cleavage.

Prior to microinjection of the titered and diluted (if required) virus stock, the cumulus

20   cell layer can be opened to provide access to the perivitelline space. The cumulus cell layer need not be completely removed from the oocyte and indeed for certain species of animals (*e.g.*, cows, sheep, pigs, or mice), a portion of the cumulus cell layer remains in contact with the oocyte to permit proper development and fertilization post-injection. Injection of viral particles into the perivitelline space allows the vector RNA (*i.e.*, the viral genome) to enter

25   the cell through the plasma membrane thereby allowing proper reverse transcription of the viral RNA. The presence of the retroviral genome in cells (*e.g.*, oocytes or embryos) infected with pseudotyped retrovirus can be detected using a variety of means, such as those described herein or as otherwise known to the skilled artisan

In an exemplary embodiment, the mutant $p27^{Kip1}$ gene can be introduced into avian

30   species using a viral vector as described in U.S. Patent No. 5,162,215 (the disclosure of which is incorporated by reference herein in its entirety). Briefly, a vector, such as a retroviral vector, is used to introduce a mutant $p27^{Kip1}$ gene into cells of an avian embryo, such as a chicken. In one embodiment, a mutant $p27^{Kip1}$ viral vector is microinjected in a newly laid chicken egg arrested at stage X (not generally more than seven days old,

unincubated), in close proximity to (*e.g.*, directly underneath) the blastoderm. More specifically, an opening about 5 mm in diameter is made in the side of the egg, normally by the use of a drilling tool fitted with an abrasive rotating tip which can drill a hole in the egg shell without damaging the underlying shell membrane. The membrane is then cut out by

5   use of a scalpel or 18 gauge needle and thumb forceps, so that a portion of the shell and membrane is removed, thereby exposing the embryo. The embryo is visualized by eye or with an optical dissecting microscope (*e.g.*, having 6X-50X magnification). A solution, usually tissue culture medium, containing the mutant $p27^{Kip1}$ gene expression vector, is microinjected into an area beneath and around the blastoderm, using a micro-manipulator

10   and a very small diameter needle (*e.g.*, glass needle about 40-60 μM outer diameter at the tip, 1 mm outer diameter along its length). The volume of solution for microinjection is typically about 5-20 μl. After microinjection, the egg is sealed with shell membrane and a sealing material, such as glue or paraffin. The sealed egg can then be incubated at approximately 38°C for various time periods up to and including the time of hatching to

15   allow normal embryo growth and development. DNA from embryos and from newly hatched chicks can be tested for the presence of the mutant $p27^{Kip1}$ gene. The presence of the mutant $p27^{Kip1}$ gene can be detected by means known in the art and appropriate to the detection of a mutant $p27^{Kip1}$ gene or gene product.

Alternatively, a mutant $p27^{Kip1}$ gene expression vector or transfected cells producing

20   the expression vector (*e.g.*, a virus containing the mutant $p27^{Kip1}$ gene) is injected into developing avian oocytes *in vivo*, for example, as described in Shuman and Shoffner (*Poultry Science* 65:1437-44 (1986), which is incorporated by reference herein in its entirety).

The overall efficiency of the nucleic acid delivery procedure to avian cells can

25   depend on the methods and timing of gene delivery. Infection efficiency is optionally increased by, for example, subjecting the blastoderm or cells derived from the blastoderm to several rounds of infection or adding a selectable marker to the mutant $p27^{Kip1}$ gene and infusing the antibiotic into the yolk or testes following transfection or cell transfer.

In another embodiment, a transgenic animal is prepared by nuclear transfer. The

30   terms "nuclear transfer" or "nuclear transplantation" refer to methods of preparing transgenic animals wherein the nucleus from a donor cell is transplanted into an enucleated oocyte. Nuclear transfer techniques or nuclear transplantation techniques are known in the art. (*See, e.g.*, Campbell *et al.*, *Theriogenology* 43:181 (1995); Collas and Barnes, *Mol. Reprod. Dev.*

38:264-67 (1994); Keefer *et al.*, *Biol. Reprod.* 50:935-39 (1994); Sims *et al.*, *Proc. Natl. Acad. Sci. USA* 90:6143-47 (1993); Prather *et al.*, *Biol. Reprod.* 37:59-86 (1988); Roble *et al.*, *J. Anim. Sci.* 64:642-64 (1987); International Patent Publications WO 90/03432, WO 94/24274, and WO 94/26884; U.S. Patent Nos. 4,994,384 and 5,057,420; the disclosures of

5   which are incorporated by reference herein in their entirety.) For example, nuclei of transgenic embryos, pluripotent cells, totipotent cells, embryonic stem cells, germ cells, fetal cells or adult cells can be transplanted into enucleated oocytes, each of which is thereafter cultured to the blastocyst stage. (As used herein, the term "enucleated" refers to cells from which the nucleus has been removed as well as to cells in which the nucleus has been

10   rendered functionally inactive.) The nucleus containing a mutant $p27^{Kip1}$ gene can be introduced into these cells by any method known to the skilled artisan, including those described herein. The transgenic cell is then typically cultured *in vitro* to the form a pre-implantation embryo, which can be implanted in a suitable female (*e.g.*, a pseudo-pregnant female).

15   The transgenic embryos optionally can be subjected, or resubjected, to another round of nuclear transplantation. Additional rounds of nuclear transplantation cloning can be useful when the original transferred nucleus is from an adult cell (*i.e.*, fibroblasts or other highly or terminally differentiated cell) to produce healthy transgenic animals.

Other methods for producing a mutant $p27^{Kip1}$ animal include methods adapted to use

20   male sperm cells to carry the mutant $p27^{Kip1}$ gene to an egg. In one example, a mutant $p27^{Kip1}$ gene can be administered to a male animal's testis *in vivo* by direct delivery. The mutant $p27^{Kip1}$ gene can be introduced into the seminiferous tubules, into the rete testis, into the vas efferens or vasa efferentia, using, for example, a micropipette. To ensure a steady infusion of the gene delivery mixture, the injection can be made through the micropipette

25   with the aid of a picopump delivering a precise measured volume under controlled amounts of pressure.

The micropipette is made of a suitable material, such as metal or glass, and is usually made from glass tubing which has been drawn to a fine bore at its working tip. The tip can be angulated in a convenient manner to facilitate its entry into the testicular tubule system.

30   Also, the micropipette can be provided with a beveled working end to allow a better and less damaging penetration of the fine tubules at the injection site. The diameter of the pipette tip is typically about 15 to 45 microns, although other sizes can be used, as needed, depending on the animal's size. The tip of the pipette can be introduced into the rete testis or the tubule system of the testicle with the aid of a binocular microscope with coaxial illumination, with

care taken not to damage the wall of the tubule opposite the injection point, and keeping trauma to a minimum. A small amount of a suitable, non-toxic dye can optionally be added to the gene delivery mixture (fluid) to confirm delivery and dissemination to the seminiferous tubules of the testis. In this manner, the gene delivery mixture reaches and is

5   brought into intimate contact with the male germ cells. Suitable male germ cells include spermatozoa (e.g., male gametes) and developmental precursors thereof.

Alternatively, the mutant $p27^{Kip1}$ gene can be introduced ex vivo into the genome of male germ cells. A number of known gene delivery methods can be used for the uptake of nucleic acid sequences into the cell. Suitable methods for introducing mutant $p27^{Kip1}$ genes

10   into male germ cells include, for example, liposomes, retroviral vectors, adenoviral vectors, adenovirus-enhanced gene delivery systems, or combinations thereof. Whether introduced in vivo or in vitro, the mutant $p27^{Kip1}$ gene, once in contact with the male germ cells, is taken up and transported into the appropriate cell location for integration into the genome and expression.

15   For ex vivo introduction of a mutant $p27^{Kip1}$ gene into the genome of an animal, male germ cells are typically obtained or collected from the donor male animal by means known in the art. The germ cells are then exposed to the mutant $p27^{Kip1}$ genes. In one exemplary embodiment, male germ cells are obtained from a donor animal by transection of the testes. Transection of the isolated testicular tissue can be accomplished, for example, by isolation of

20   the animal's testes, decapsulation, teasing apart and mincing of the seminiferous tubules. The separated cells can then be incubated in an enzyme mixture comprising enzymes to gently break up the tissue matrix and release undamaged cells such as, for example, pancreatic trypsin, collagenase type I, pancreatic DNase type I, as well as bovine serum albumin, in a modified DMEM medium. The cells can be incubated in the enzyme mixture

25   for a period of about 5 minutes to about 30 minutes, more typically about 15 minutes to about 20 minutes, at a temperature of about 33°C to about 37°C. After washing the cells free of the enzyme mixture, they can be placed in an incubation medium, such as DMEM, and plated on a culture dish for exposure to mutant $p27^{Kip1}$ genes.

A typical method of isolating or selecting male germ cell populations comprises

30   obtaining specific male germ cell populations, such as spermatogonia, from a mixed population of testicular cells by extruding the cells from the seminiferous tubules and gentle enzymatic disaggregation. The spermatogonia or other male germ cell populations can be isolated from a mixed cell population by a method including the utilization of a promoter sequence, which is specifically or selectively active in cycling male germ line stem cell

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populations, as disclosed in International Patent Publication WO 0069257 (the disclosure of which is incorporated by reference herein in its entirety).

After transfer to the testes of a male animal, further selection can be preformed after biopsy of one or both of the recipient male's testes, or after examination of the animal's

5   ejaculate to confirm whether the mutant p27^{Kip1} gene was incorporated (*e.g.*, by the polymerase chain reaction). The initial gene delivery can optionally include a positively selectable marker, such as a gene encoding the Green Fluorescent Protein, enhanced Green Fluorescent Protein (EGFP), Yellow Fluorescent Protein, Blue Fluorescent Protein, a phycobiliprotein, such as phycoerythrin or phycocyanin, or any other selectable marker

10   which fluoresces under light of suitable wave-lengths, or encoding a light-emitting protein, or is other detectable.

In certain embodiments, the male germ cells containing a mutant p27^{Kip1} gene can be introduced into one or more of the testes of the recipient male vertebrate after the testes of the recipient animal are depopulated of native germ cells. Substantial depopulation of the

15   endogenous male germ cells facilitates the colonization of the recipient testis by the mutant p27^{Kip1} germ cells. Depopulation of the testes can be done by any suitable means, including, for example, by gamma irradiation, by chemical treatment, by means of infectious agents such as viruses, by autoimmune depletion, or by combinations thereof. In certain embodiments, the testes are depopulated by combined treatment with an alkylating agent and

20   gamma irradiation. The alkylating agent can be, for example, busulfan (1,4-butanediol dimethanesulphonate; Myleran, Glaxo Wellcome), chlorambucil, cyclophosphamide, melphalan, or ethyl ethanesulfonic acid, combined with gamma irradiation. A typical dose of alkylating agent is about 4 to 10 milligrams per kilogram of body weight. (*See, e.g.,* International Patent Publication WO 00/69257, the disclosure of which is incorporated by

25   reference herein in its entirety.) The alkylating agent can be administered by any pharmaceutically acceptable delivery system, including but not limited to, intraperitoneal, intravenous, or intramuscular injection, intravenous drip, implantation, transdermal or transmucosal delivery systems. The recipient animal can be gamma irradiated with a dose, for example, of about 200 to about 800 Rads, or about 350 to 450 Rads, directed locally to

30   the testis to be depopulated.

During depopulation, the basic rigid architecture of the gonad is usually not destroyed, nor badly damaged. If there is disruption of the fine system of tubule formation, it can be difficult for the exogenous spermatogonia to repopulate the testis. Disruption of tubules might also lead to impaired transport of testicular sperm and result in infertility. Any

controlled testicular injury of this kind is usually limited so that the Sertoli cells are not irreversibly damaged, as they are needed to provide a base for development of the germ cells during maturation. Moreover, they may play a role in preventing the host immune defense system from destroying grafted foreign spermatogonia.

5   Transferring the treated germ cells into the recipient testis can be accomplished by direct injection using a suitable micropipette. Support cells, such as Leydig or Sertoli cells that provide hormonal stimulus to spermatogonial differentiation, can be transferred to a recipient testis along with the modified germ cells. These transferred support cells can be autologous or heterologous to either the donor or recipient testis. A suitable concentration of

10   cells in the transfer fluid can easily be established by simple experimentation, and in certain embodiments can be within the range of about 1×10^5 to about 1×10^6 cells per 10 μl of fluid. These cells can be introduced into the vasa efferentia, the rete testis or the seminiferous tubules, optionally with the aid of a picopump to control pressure and/or volume. Alternatively, the delivery can be performed manually. The micropipette employed

15   is in most respects similar to that used for the *in vivo* injection (as described *supra*), except that its tip diameter generally will be about 45 to about 70 microns.

          Alternatively, for avian transgenic animals, the testes can be repopulated by using blastoderm removed from an avian egg. The blastoderm cells can be pooled with cells from several eggs, as needed. *In vitro*, nucleic acid uptake by blastodermal cells can be facilitated

20   by such techniques as electroporation, DEAE-dextran treatment, calcium phosphate treatment, lipofection, and the like. Following transfection, the cells can be transferred into the testes of a rooster (*e.g.*, a sterile rooster) to induce development in spermatogonia and sperm for breeding.

          The present invention also provides animal semen containing a plurality of male

25   mutant p27^{Kip1} germ cells, which is useful for breeding or other suitable purposes. The semen is obtained from ejaculate produced by mutant p27^{Kip1} transgenic male animals or their transgenic male progeny (either immediate progeny or progeny separated by one or more generations). Methods of inducing ejaculation by a male animal and capturing the semen are well known. The semen can be processed (*e.g.*, by washing, and/or stored) by

30   means such as are known in the art. For example, storage conditions include the use of cryopreservation using programmed freezing methods and/or the use of cryoprotectants, such as, for example, dimethyl sulfoxide (DMSO), glycerol, trehalose, or propanediol-sucrose, and storage in substances such as liquid nitrogen. Cryopreservation is useful for

transport of gametes as frozen germ cells. Such transport can facilitate the establishment of various valued livestock, fowl lines, and the like, at a remote distance from the donor animal.

Following transfer of a mutant p27^{Kip1} gene to male germ cells by any suitable method, a transgenic zygote can be formed by breeding the male animal with a female

5 animal. The transgenic zygote can be formed, for example, by natural mating (*e.g.*, copulation by the male and female vertebrates of the same species), or by *in vitro* or *in vivo* artificial means. Suitable artificial means include, but are not limited to, artificial insemination, *in vitro* fertilization (IVF) and/or other artificial reproductive technologies, such as intracytoplasmic sperm injection (ICSI), subzonal insemination (SUZI), partial zona

10 dissection (PZD), and the like, as will be appreciated by the skilled artisan. (*See, e.g.*, International Patent Publication WO 00/09674, the disclosure of which is incorporated by reference herein in its entirety.)

A variety of methods can be used to detect the presence of mutant p27^{Kip1} genes in target cells and/or transgenic animals. Since the frequency of transgene incorporation (*i.e.*,

15 mutant p27^{Kip1} gene) can be low, although reliable, the detection of transgene integration in the pre-implantation embryo can be desirable. In one aspect, embryos are screened to permit the identification of suitable mutant p27^{Kip1} embryos for implantation to form transgenic animals. For example, one or more cells are removed from the pre-implantation embryo. When equal division of the embryo is used, the embryo is typically not cultivated past the

20 morula stage (32 cells). Division of the pre-implantation embryo (reviewed by Williams *et al.*, *Theriogenology* 22:521-31 (1986)) results in two "hemi-embryos" (hemi-morula or hemi-blastocyst), one of which is capable of subsequent development after implantation into the appropriate female to develop in utero to term. Although equal division of the pre-implantation embryo is typical, it is to be understood that such an embryo can be unequally

25 divided either intentionally or unintentionally into two hemi-embryos. Essentially, one of the embryos which is not analyzed usually has a sufficient cell number to develop to full term *in utero*. In a specific embodiment, the hemi-embryo (which is not analyzed), if shown to be transgenic, can be used to generate a clonal population of transgenic animals, such as by embryo splitting.

30 One of the hemi-embryos formed by division of pre-implantation embryos can be analyzed to determine if the mutant p27^{Kip1} gene has integrated into the genome of the organism. Each of the other hemi-embryos can be maintained for subsequent implantation into a recipient female, typically of the same species. A typical method for detecting a mutant p27^{Kip1} gene at this early stage in the embryo's development uses these hemi-

embryos in connection with allele-specific PCR, which can differentiate between a mutant p27^{Kip1} gene and a wildtype p27^{Kip1} gene. (*See, e.g.,* McPherson *et al.* (eds) *PCR2: A Practical Approach,* Oxford University Press (1995); Cha *et al., PCR Methods Appl.* 2:14-20 (1992); the disclosures of which are incorporated by reference herein.)

5   After a hemi-embryo is identified as a transgenic hemi-embryo, it optionally can be cloned. Such embryo cloning can be performed by several different approaches. In one cloning method, the transgenic hemi-embryo can be cultured in the same or in a similar media as used to culture individual oocytes to the pre-implantation stage. The "transgenic embryo" so formed (typically a transgenic morula) can then be divided into "transgenic

10   hemi-embryos" which can be implanted into a recipient female to form a clonal population of two transgenic non-human animals. Alternatively, the two transgenic hemi-embryos obtained can be again cultivated to the pre-implantation stage, divided, and recultivated to the transgenic embryo stage. This procedure can be repeated until the desired number of clonal transgenic embryos having the same genotype are obtained. Such transgenic embryos

15   can then be implanted into recipient females to produce a clonal population of transgenic non-human animals.

In addition to the foregoing methods for detecting the presence of a mutant p27^{Kip1} gene, other methods can be used. Such methods include, for example, *in utero* and post partum analysis of tissue. *In utero* analysis can be performed by several techniques. In one,

20   transvaginal puncture of the amniotic cavity is performed under echoscopic guidance (*see, e.g.,* Bowgso *et al., Bet. Res.* 96:124-27 (1975); Rumsey *et al., J. Anim. Sci.* 39:386-91 (1974)). This involves recovering amniotic fluid during gestation. Most of the cells in the amniotic fluid are dead. Such cells, however, contain genomic DNA which can be subjected to analysis (*e.g.,* by PCR) for the mutant p27^{Kip1} gene as an indication of a successful

25   transgenesis. Alternatively, fetal cells can be recovered by chorion puncture. This method also can be performed transvaginally and under echoscopic guidance. In this method, a needle can be used to puncture the recipient animal's placenta, particularly the placentonal structures, which are fixed against the vaginal wall. Chorion cells, if necessary, can be separated from maternal tissue and subjected to PCR analysis for the mutant p27^{Kip1} gene as

30   an indication of successful transgenesis.

The presence of a mutant p27^{Kip1} gene can also be detected after birth. In such cases, the presence of a mutant p27^{Kip1} gene can be detected by taking an appropriate tissue biopsy, such as from an ear or tail of the putative transgenic animal. The presence of a mutant

p27^{Kip1} gene can also be detected by assaying for expression of the mutant p27^{Kip1} polypeptide in a tissue.

The location and number of integration events can be determined by methods known to the skilled artisan. (*See, e.g.*, Ausubel *et al.*, *supra*; Sambrook *et al.*, *supra*.) For example, PCR or Southern blot analysis of genomic DNA extracted from infected oocytes and/or the resulting embryos, offspring and tissues derived therefrom, can be employed when information concerning site of integration of the viral DNA into the host genome is desired. To examine the number of integration sites present in the host genome, the extracted genomic DNA can typically be digested with a restriction enzyme which cuts at least once within the vector sequences. If the enzyme chosen cuts twice within the vector sequences, a band of known (i.e., predictable) size is generated in addition to two fragments of novel length which can be detected using appropriate probes.

Other methods of preparing transgenic animals are disclosed, for example, in U.S. Patent Nos. 5,633,076 or 6,080,912; and in International Patent Publications WO 97/47739, WO 99/37143, WO 00/75300, WO 00/56932, and WO 00/08132, the disclosures of which are incorporated herein by reference in their entirety.

EXAMPLES

The present invention can be illustrated by the following Examples. These examples illustrate principles of the present invention and are not intended to limit the scope of the invention.

Example 1

In this example, the effect of an amino acid substitution, threonine 187 to alanine in a mouse p27^{Kip1} gene, on mice was studied.

Methods

Mice

To construct the genomic targeting vector, a 5.9 kilobase (kb) *Bam* HI fragment was isolated from a 17 kb *Not* I fragment which contains the entire coding region of the p27^{Kip1} gene obtained from a mouse 129/Sv 1 genomic library (as described by Fero *et al.*, *Cell* 85:733-44 (1996)). Codon 187 of exon 2, which encoded threonine, was mutated to alanine site directed mutagenesis (acg→gcg) to make the p27^{T187A} allele. A 3 kb *Sac* I fragment

containing an antibiotic resistance expression cassette (comprising a *pgk* promoter driving expression of a neomycin resistance gene followed by a transcription termination) was isolated as a *Bam* HI/*Hind* III fragment from pBS302 (Gibco BRL). Nucleic acids encoding *loxP* sequences were attached to each end of an antibiotic resistance expression cassette.

5   The modified expression cassette was inserted into a *Sac* I site in the p27^{Kip1} promoter of the 5.9 kb *Bam* HI p27^{Kip1} gene fragment (in the antisense orientation) to create the construct p27T187A 5.9 Neo/STOP. This construct was then cloned into the pPNT vector (Fero *et al.*, *Cell* 85:733-44 (1996)), thus creating the genomic targeting vector.

For construction of mouse embryonic stem cells containing the p27T187A 5.9
10   Neo/STOP construct, the targeting vector was linearized with *Not* I and transduced by electroporation into mouse XY AK7 embryonic stem (ES) cells (Friedrich *et al.*, *Genes & Development* 5:1513-23 (1991)). Transduced embryonic stem cells were selected in 400 μg/ml G418 and 0.4 μM FIAU. Neomycin resistant colonies of ES cells were screened for homologous recombination of the p27T187A 5.9 Neo/STOP construct at the p27^{Kip1} locus by
15   Southern blotting using a probe external to the 5' end of the targeting construct. In all 5 ES cell clones used for blastocyst injection, integration of the T187A mutation was verified by DNA sequence analysis. Transduced ES cells containing the homologously integrated p27T187A 5.9 Neo/STOP were designated p27^{T187A} ES cells.

p27^{T187A} ES cells were introduced by microinjection into 5 dpc C57/B6J mouse
20   embryos. Germline transmission of the p27T187A 5.9 Neo/STOP construct was identified in male chimeras representing three separate ES cell clones.

To excise the neo/STOP cassette from the p27^{Kip1} gene, chimeric male mice were bred with female CMV-cre transgenic mice (TgN (CMV-Cre)1AN) (Nagy *et al.*, *Curr. Biol.* 8:661-64 (1998)). Excision of the neo/STOP cassette was verified by PCR using primers
25   derived from the p27^{T187A} genomic sequence upstream of the *Sac* I site (Y1, GAGCAGGTTTGTTG GCAGTCGTACACCTCC) (SEQ ID NO:1), from the neomycin gene (A4, CGTGGGATCATTGT TTTTCTCTTG) (SEQ ID NO:2), and from genomic sequence downstream of the *Sac* I site (H3, CCAATATGGCGGTGGAAGGGAGGCTGA) (SEQ ID NO:3). Homozygous integration of the T187A mutation was confirmed by the
30   presence of a 34 base pair (bp) loxP site insertion into the wildtype 0.25 kb PCR fragment using primers Y1 and H3.

Mouse Embryonic Fibroblasts

35

p27^{T187A} heterozygous males and females were crossed and embryos were dissected 12.5 –13.5 days after detection of vaginal plugs. The head and internal organs were removed, and the embryos were minced and incubated in 0.05% trypsin for 5 minutes. The cells were resuspended in Dulbecco's modified Eagle medium (DMEM) supplemented with

5   10% FBS. After centrifugation, the supernatant was discarded, and the cell suspension from each embryo was cultivated on a 10-cm dish in 8 ml of DMEM with 10% FBS until confluency was reached. After this time, the cells were trypsinized, counted and plated at 1.4x10^6 cells/10-cm dish every three days.

10   Cell Culture

Passage 2-3 mouse embryonic fibroblasts (MEFs) were plated as 1.4x10^6 cells/10-cm dish grown in DMEM with 10% FBS for 3 days after which the media was removed, the plates washed with PBS, and the cells incubated in DMEM containing 0.1% FBS for 72 hours. The cells were then washed with PBS, trypsinized, counted and resuspended in

15   DMEM containing 10% FBS at 1.4x10^6 cells/10-cm dish and 0.5x10^5 cells/6-cm dish. For each time point, the cells were labeled with 10μM BrdU (Sigma) for 30 minutes, scraped off the plate, washed with PBS and fixed in 70% ethanol for at least 24 hours. Nuclei were purified and labeled with 100 μl anti BrdU-FITC antibody (Pharmingen) as previously described (White *et al.*, *Cytometry* 11:314-17 (1990)). After this incubation nuclei were

20   treated with RNAse A, counterstained with propidium iodide (100 μg/ml) and analyzed on a Becton Dickinson Flow Cytometer using Becton Dickinson Cell Quest software.

To separate G1 from S phase cells, cells were labeled with Hoechst Stain (Sigma) (10 μg/ml) for 30 minutes, trypsinized and separated on a Vantage SE flow cytometer (BD systems) using Cell Quest software. For p27^{Kip1} half life measurements, cycloheximide

25   (chx) (10 μg/ml final concentration) was added to the cells at the indicated times. p27^{Kip1} protein levels were determined by immunoblotting. The resulting autoradiograms were scanned and the intensity of the p27^{Kip1} bands quantitated using Image Quant software (Molecular Dynamics) and normalized to actin controls.

30   Isolation and stimulation of T lymphocytes

Splenic CD4$^+$ T cells were purified following red cell lysis (Whole Blood Erythrocyte Lysing Kit, R&D systems). Mononuclear cells were enriched using ficoll gradient centrifugation. CD4$^+$ T cells were isolated using the mouse T Cell CD4 Subset

Columns (R&D Systems). $CD4^+$ T cells were activated with plate-bound anti-CD3 antibody without or with the addition of recombinant mouse IL-2 (Pharmingen) (*i.e.*, 0, 10 Units of IL-2/ml or 100 Units of IL-2/ml). 72 hours after stimulation T cells were labeled with [^{3}H] thymidine for 4 hours, harvested and the incorporation of radioactivity into DNA

5   determined. All measurements were done in triplicate.

Immunoblotting

The antibodies used for immunoblotting were mouse monoclonal anti-p27^{Kip1} (Transduction Laboratories), rabbit polyclonal anti-cyclin A (Santa Cruz Biotechnology),

10   anti-Actin (Santa Cruz), and anti-Cdk2 (Santa Cruz).

Wound Healing

Full thickness punch wounds (4 mm) were applied as previously described (Subramaniam *et al.*, *Amer. J. Path.* 150:1701-09 (1997)). Animals were injected with BrdU

15   (1 mg/ml, 30 μl/g) 16 hours before they were sacrificed. Wounds were excised, fixed and embedded in paraffin 4.5 days after wounding (Kyriakides *et al.*, *J. Invest. Dermatol.* 113:782-87 (1999)). Wounds were stained with anti-BrdU Ab (AB1, NeoMarkers). BrdU staining was visualized using the Darko Ark Kit and counterstained with hematoxylin and eosin. Wound sizes and epithelial gap diameters were determined by optical micrometer

20   measurements using 100x magnification.

Results

In this example, the role of T187 phosphorylation in determining p27^{Kip1} protein abundance, and in controlling cell proliferation, was examined by examining wildtype mice

25   (having unmutated p27^{Kip1}) and p27^{T187A} mice. (p27^{T187A} mice express a non-phosphorylatable form of p27^{Kip1} in which the conserved threonine at position 187 was changed to alanine.) p27^{T187A} mice were prepared by precisely replacing the wildtype p27^{Kip1} gene with the p27^{T187A} allele. Previous experiments using cultured fibroblasts had shown that ectopic over-expression of p27^{T187A} imposed an irreversible G1 arrest (Sheaff *et*

30   *al.*, *Genes & Development* 11:1464-78 (1997)). Thus, it was expected that over-expression of p27^{T187A} in mice would also impose an irreversible G1 arrest on cells harboring this mutant allele. To prevent this G1 block to cell replication, a 'lox-STOP-lox' element was placed within the promoter of the p27^{T187A} allele so that the mutant allele could be conditionally activated with the Cre recombinase.

Mice heterozygous for the p27^{T187A} allele (containing the lox-STOP-lox construct) were bred to mice that constitutively express Cre recombinase in the germline, which resulted in deletion of the lox-STOP-lox element. The p27^{T187A} (Δlox-STOP-lox) allele was bred to homozygosity and shown to express the p27^{T187A} protein at levels equivalent to the

5   wildtype allele in all tissues. Control experiments showed that p27^{T187A} mice and wildtype p27^{Kip1} mice were equally able to inhibit Cdk2 *in vitro* kinase activity when either histone H1 or the retinoblastoma protein was used as a substrate (Sheaff *et al., Genes & Development* 11:1464-78 (1997)). Therefore, the T187A substitution did not produce an intrinsic change in its molecular properties as a Cdk inhibitor. Surprisingly, expression of

10 the p27^{T187A} allele did not affect viability or fertility, and thus did not produce the expected block in G1 replication. Consequently, all further experiments were performed on mice homozygous for the p27^{T187A} allele, and which did not contain the Cre recombinase transgene.

The effect of the T187A amino acid substitution on regulation of the p27^{Kip1} protein

15 was determined by comparing the protein levels of p27 and p27^{T187A} in mouse embryonic fibroblasts (MEFs) that were made quiescent by serum deprivation and then stimulated to synchronously enter the cell cycle by readdition of serum. The half lives of p27 and p27^{T187A} were measured in G0, G1 and S phase MEFs. Cells were synchronized by serum starvation (G0) and refeeding (G1 = 12 hours post refeeding; S = 24 hours post refeeding).

20 p27 protein levels were determined by immunoblotting of cell extracts from cells synchronized through two cell cycles. Control MEFs were synchronized by serum starvation and then stimulated to enter the cell cycle by refeeding with serum. One group of cells was allowed to proceed through G1, while the other group was treated with the proteasome inhibitor MG-132 (10 μM) at 6 hours post serum stimulation. p27 protein levels were then

25 measured at the indicated time points by immunoblotting. Quiescent Skp2 +/+ and skp2 -/- MEFs were stimulated to re-enter the cell cycle by addition of serum, and p27 protein levels were determined at the indicated time points by immunoblotting.

In control MEFs, p27 protein levels declined to low levels between 12 and 15 hours after serum stimulation. This time corresponded to the early/mid-G1 part of the cell cycle.

30 p27 protein levels remained at low levels for the duration of the cell cycle. p27^{T187A} was expressed at the same level as wildtype p27 in quiescent MEFs. This observation is consistent with the observation that p27 and p27^{T187A} were expressed at equal levels in mouse tissues *in vivo* (and which are composed largely of non-dividing cells). Serum stimulation of MEF's containing p27^{T187A} caused the p27^{T187A} protein levels to decline with

kinetics similar to those of wildtype p27. However, in contrast to the wildtype protein, p27^{T187A} protein then re-accumulated as cells completed G1 and entered S phase. Indeed, in late S/G2 the amount of p27^{T187A} rose to a level that was similar to its abundance in quiescent cells. This increase in abundance was associated with an increased amount of p27

5   bound to cyclin A and a 50% reduction in cyclin A-associated kinase activity. There was no change in total cyclin A protein levels, and the length of S phase was not altered. These studies demonstrated that p27 was down-regulated in a T187-dependent manner in S and G2, and independently of T187 in G1.

          The absence of the T187-dependent pathway for p27 turnover had significant effects

10   on cell proliferation in various cells and tissues of the p27^{T187A} mouse. In general, rising levels of p27^{T187A}, which occurred in late G1/S/G2 cells, created a barrier to cell cycle progression. The severity of the ensuing proliferation defect varied among different cell types, however. A modest effect was seen in MEFs, where expression of p27^{T187A} caused a 20-30% reduction in the number of cells which entered S phase after serum stimulation.

15   This result was later confirmed using three independently isolated MEF strains from three different founder mice.

          A relatively greater defect was seen when purified CD4$^+$ splenic T lymphocytes were stimulated to proliferate with antibodies directed against the T cell antigen receptor. DNA replication was reduced by 80% in cells expressing p27^{T187A} compared to control T cells.

20   Addition of exogenous IL-2 partially restored proliferation of the cells expressing p27^{T187A}, suggesting that high levels of IL-2 might promote a T187 independent pathway for decreasing p27.

          A defect in cell proliferation was also observed in dermal keratinocytes expressing p27^{T187A}. Keratinocyte proliferation was induced *in vivo* by creating circular, 4 mm full

25   thickness punch wounds in the skin overlying the scapula and extending through the epidermis and dermis. The rate of healing was monitored by gross inspection and by histological examination at 4.5 days after wounding. This analysis revealed a delay in wound re-epithelialisation in the p27^{T187A} mice. The epithelial gap measured as the distance between the keratinocyte edges growing into the woundbed made up 60% (±5) of the entire

30   wound in the p27^{T187A} mice as compared to 35% (±9) in the control mice (n=12). This difference was most likely a result of an impaired proliferative response, because p27^{T187A} keratinocytes at the wound edge displayed reduced levels of BrdU incorporation (13.5% (±8.5) p27^{T187A} versus 35% (±5) control). No difference was observed in the healing of

incisional wounds in $p27^{T187A}$ versus control mice, which occurs mostly by epithelial cell migration rather than proliferation.

Surprisingly, despite the restraint on cell proliferation created by the $p27^{T187A}$ mutant protein, mice expressing this protein developed normally and attained an average size that was even larger than wildtype mice. Growth curves for female $p27^{Kip1}$ +/+, p27 -/- (homozygous null alleles) and $p27^{T187A}/p27^{T187A}$ mice were prepared. An average of 30 mice was observed of each type. The $p27^{Kip1}$ +/+ and $p27^{T187A}/p27^{T187A}$ mice were littermates (F2 hybrids B6/C57 x 129/Sv). Weight data for the p27 null mice were obtained from an earlier study, which used mice of the same genetic background as those used here (Fero *et al.*, *Cell* 85:733-44 (1996)).

One possibility was that the T187A substitution had partially disabled $p27^{Kip1}$ function, resulting in cellular hyperplasia similar to that seen in the $p27^{Kip1}$ knockout mouse. This possibility was examined in the thymus of the $p27^{T187A}$ mice, which like all other organs was enlarged in proportion to overall body size. In contrast to the results observed in $p27^{Kip1}$ knockout mice (Fero *et al.*, *Nature* 396:177-80 (1998); Nakayama *et al.*, *Cell* 85:707-20 (1996); Kiyokawa *et al.*, *Cell* 85:721-32 (1996)), mice expressing $p27^{T187A}$ did not show an increased amount of cell proliferation, as determined by BrdU labeling. This result indicates that the T187A substitution and the $p27^{Kip1}$ gene deletion affected organ size by different mechanisms. Further, other phenotypes associated with $p27^{Kip1}$ deficiency were not seen in the mice expressing $p27^{T187A}$, including female sterility, pituitary tumorigenesis, and disrupted retinal architecture.

These results showed that $p27^{Kip1}$ abundance is controlled by two different mechanisms, the first acting in early/mid G1 cells and the second in late G1, S and G2. The increased turnover of $p27^{Kip1}$ protein was the mechanism underlying not only the T187 pathway for $p27^{Kip1}$ regulation, but the earlier G1 pathway as well. In quiescent cells, $p27^{Kip1}$ was relatively stable with a half life of 10-12 hours. Serum stimulation decreased $p27^{Kip1}$ stability, reducing its half life to approximately 2 hours in both G1 and S phase cells. $p27^{T187A}$ was also stable in quiescent cells, and after serum stimulation became unstable in mid-G1 similar to the wildtype protein. In S phase cells, however, $p27^{T187A}$ became stable again, acquiring a long half life very similar to what it had been in quiescent, mitogen starved cells. Thus, rapid turnover of $p27^{Kip1}$ in S phase cells requires T187, whereas the rapid turnover of $p27^{Kip1}$ in G1 cells does not. These results also implied that the proteolytic pathway which degraded $p27^{Kip1}$ in G1 cells was not operative in S phase.

This G1-specific turnover pathway for p27^{Kip1} is not a unique feature of cells as they exit quiescence, but rather occurs during each mitotic cycle. Quiescent MEFs were stimulated with serum mitogens for 18 hours at which time they were separated by flow cytometry into G1 and S phase populations. As seen previously, p27^{Kip1} protein levels were

5 lower in G1 cells than in quiescent cells. In the S phase population, however, the abundance of wildtype p27^{Kip1} declined further whereas the opposite occurred in MEFs expressing p27^{T187A} polypeptide. The S phase cells were then replated and allowed to progress through the division cycle until a time when 80% of the cells had entered the next G1 phase. The abundance of p27^{T187A} declined again in the second G1 just as it had in the first G1,

10 demonstrating the periodic nature of the G1-turnover pathway.

Phosphorylation of p27^{Kip1} on T187 is known to trigger its ubiquitination by the Skp2-containing SCF E3 complex, and its subsequent turnover in the proteasome (Sheaff *et al.*, *Genes & Development* 11:1464-78 (1997); Vlach *et al.*, *EMBO J.* 15:6595-604 (1996); Muller *et al.*, *Oncogene* 15:2561-76 (1997); Sutterluty *et al.*, *Nature Cell Biol.* 1:207-14

15 (1999); Rolfe *et al.*, *J. Mol. Med.* 75:5-17 (1997); Carrano *et al.*, *Nature Cell Biol.* 1:193-99 (1999); Tsvetkov *et al.*, *Curr. Biol.* 9:661-64 (1999)). The turnover of p27^{Kip1} in G1 cells was also proteosome and Skp2-dependent. Quiescent, serum starved MEFs were re-stimulated with serum and six hours later treated with MG132, an inhibitor of proteasomal proteolysis. This prevented the normal decrease in p27^{Kip1} protein levels that occurs in mid-

20 G1. Furthermore, p27^{Kip1} protein levels did not decline, either in G1 or in S phase, in serum stimulated skp2 null MEFs. These cells presumably continue to proliferate because Skp2 is also needed for degradation of cyclin E31. Therefore, although the trigger for p27^{Kip1} turnover is different in G1 versus S phase, both pathways ultimately lead to the degradation of p27^{Kip1} by Skp2- and proteosome-dependent mechanisms.

25 These data show that T187-dependent turnover of p27^{Kip1} is important for normal regulation of p27^{Kip1} and normal control of cell division. However, contrary to the expected results, inactivating this pathway has neither a universal nor severe effect on cell proliferation. This observation is explained, at least in part, by a previously unrecognized T187-independent pathway for p27^{Kip1} degradation that is activated during each G1 phase of

30 the cell cycle. This pathway allows many cells expressing p27^{T187A} to complete the cell cycle before the re-accumulation of p27^{Kip1} in S phase can stop it. The T187 pathway, by keeping p27^{Kip1} levels low for the duration of S and G2, allows the cell to slow its rate of progression through this part of the cell cycle (for instance, in response to DNA damage) without having to confront the rising p27^{Kip1} levels which would otherwise occur.

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Thus, two proteolytic pathways act in sequence during the cell cycle to control p27^{Kip1} abundance. The first pathway functions during early to mid G1 and is triggered by mitogens. It may be activated by Ras and Myc, and underlie the ability of these proteins to reduce p27^{Kip1} abundance and promote serum-independent entry into S phase (Leone *et al.*,

5   *Nature* 387:422-26 (1997); O'Hagan *et al.*, *Genes & Development* 14:2185-91 (2000)). Inhibition of p27^{Kip1} at the level of translation (Agrawal *et al.*, *Mol. Cell. Biol.* 16:4327-36 (1996); Hengst *et al.*, *Science* 271:1861-64 (1996); Millard *et al.*, *Mol. Cell. Biol.* 20:5947-59 (2000); Millard *et al.*, *J. Biol. Chem.* 272:7093-98 (1997)), and by sequestration into cyclin D/Cdk complexes (Sherr *et al.*, *Genes & Development* 13:1501-12 (1999)) also

10   contribute to down regulation of p27^{Kip1} during the early to mid G1 cell cycle period. Down-regulation of p27^{Kip1} by the concerted action of these pathways results in the initial production of active cyclin E-Cdk2, and consequently the onset of the second pathway for p27^{Kip1} turnover. This second pathway operates in late G1, S and G2, and is dependent upon Cdk2-mediated phosphorylation of p27^{Kip1} on T187. Once initiated, this second pathway

15   would be amplified by a self-reinforcing positive feedback loop, and therefore would continue even if the initial mitogenic stimulus were withdrawn. In this way, inactivation of p27^{Kip1} switches in mid G1 from being mitogen-dependent to being mitogen-independent, which is analogous to the consecutive mitogen-dependent and mitogen-independent pathways that inactivate Rb during the same cell cycle interval (Hatakeyama *et al.*, *Cold*

20   *Spring Harbor Symposia on Quantitative Biology* 59:1-10 (1994)). Sequentially acting pathways that inactivate key cell cycle inhibitors can be the biochemical underpinnings of the cell cycle transition from mitogen-dependence to mitogen-independence, which has been called the G1 restriction point (Pardee, *Proc. Natl. Acad. Sci. USA* 71:1286-90 (1974)).

25   The previous examples are provided to illustrate but not to limit the scope of the claimed inventions. Other variants of the inventions will be readily apparent to those of ordinary skill in the art and encompassed by the appended claims. All publications, patents, patent applications and other references cited herein are hereby incorporated by reference.

WHAT IS CLAIMED IS:

1. An isolated transgenic cell having a mutant p27^{Kip1} gene lacking a Cdk2 phosphorylation site, wherein the mutant p27^{Kip1} gene encodes a mutant p27^{Kip1} polypeptide having a longer half-life in S phase than wildtype p27^{Kip1} polypeptide.

2. The transgenic cell of claim 1, wherein the mutant p27^{Kip1} polypeptide inhibits Cdk2 *in vitro* kinase activity.

3. The transgenic cell of claim 1, wherein the mutant p27^{Kip1} polypeptide is p27^{T187A}.

4. The transgenic cell of claim 1, wherein the mutant p27^{Kip1} gene is located at an endogenous p27^{Kip1} locus.

5. The transgenic cell of claim 4, wherein the cell is heterozygous or homozygous for the mutant p27^{Kip1} gene.

6. The transgenic cell of claim 1, wherein the cell is a primordial germ cell, an oocyte, egg, spermatocyte, sperm cell, fertilized egg, zygote, or embryonic stem cell.

7. The transgenic cell of claim 6, wherein the cell is an oocyte, fertilized egg, sperm cell or spermatocyte.

8. The transgenic cell of claim 1, comprising progeny of the cell of claim 1.

9. The transgenic cell of claim 1, wherein the cell is a somatic cell.

10. A non-human, transgenic animal which comprises a nucleic acid sequence encoding a mutant p27^{Kip1} protein lacking a Cdk2 phosphorylation site.

11. The transgenic animal of claim 10, wherein the mutant p27^{Kip1} protein is p27^{T187A}.

12. The transgenic animal of claim 10, wherein the transgenic animal is a primate, mammal, bovine, porcine, ovine, equine, avian, rodent, fowl, piscine, or crustacean.

13. The transgenic animal of claim 12, wherein the transgenic animal is a farm animal.

14.   The transgenic animal of claim 13, wherein the farm animal is a chicken, cow, bull, horse, pig, sheep, goose or duck.

15.   A transgenic, non-human animal whose genome comprises a $p27^{Kip1}$ gene and expresses a mutant $p27^{Kip1}$ polypeptide having a longer half-life in S phase than wildtype p27 polypeptide, wherein the expression results in increased size or growth rate of the animal.

16.   The transgenic animal of claim 15, wherein the transgenic animal is a primate, mammal, bovine, porcine, ovine, equine, avian, rodent, fowl, piscine, or crustacean.

17.   The transgenic animal of claim 15, wherein the transgenic animal is a farm animal.

18.   The transgenic animal of claim 17, wherein the farm animal is a chicken, cow, bull, horse, pig, sheep, duck or goose.

19.   A method for increasing the size or growth rate of a non-human, transgenic animal, comprising:

stably introducing into a genome of an animal cell a mutant $p27^{Kip1}$ gene lacking a Cdk2 phosphorylation site; and

producing an animal from the animal cell.

20.   The method of claim 19, further comprising:

transferring a nucleus from the animal cell into a second cell from which an animal can be reconstituted;

and allowing the second cell to develop into an immature animal;

whereby the immature animal is larger than an immature animal not having the mutant $p27^{Kip1}$ gene.

21.   The method of claim 20, wherein the second cell is an enucleated fertilized egg.

22.   The method of claim 19, further comprising:

homologously integrating the mutant $p27^{Kip1}$ gene at an endogenous $p27^{Kip1}$ locus in the animal cell.

23.   The method of claim 19, wherein the mutant $p27^{Kip1}$ gene is heterologous to the animal cell.

24.   The method of claim 19, wherein mutant $p27^{Kip1}$ gene is integrated at a non-$p27^{Kip1}$ locus.

25.   The method of claim 19, wherein the mutant $p27^{Kip1}$ gene encodes $p27^{T187A}$.

26.   The method of claim 19, wherein the animal cell is a germ cell, a totipotent cell, a stem cell, an embryonic stem cell, a pluripotent stem cell, a somatic cell, or a fetal cell.

27.   The method of claim 26, wherein the germ cell is a primordial germ cell, oocyte, egg, spermatocyte, sperm cell, fertilized egg, zygote or blastomere.

28.   The method of claim 19, wherein the animal cell is from a vertebrate.

29.   The method of claim 28, wherein the vertebrate is a primate, mammal, bovine, porcine, ovine, equine, avian, rodent, fowl, piscine, or crustacean.

30.   The method of claim 29, wherein the vertebrate is a chicken, hen, rooster, cow, bull, duck or goose.

31.   The method of claim 19, wherein the introducing is by electroporation, microinjection, lipofection, transfection or biolistics.

32.   The method of claim 19, wherein the mutant $p27^{Kip1}$ gene comprises an expression cassette comprising a heterologous promoter operably associated with an open reading frame encoding $p27^{T187A}$ operably associated with a polyadenylation sequence.

33.   The method of claim 19, wherein the mutant $p27^{Kip1}$ gene further comprises a selectable marker.

34.   The method of claim 33, wherein the selectable marker is neo.

35.   The method of claim 19, wherein the introducing is by a viral vector.

36.   A method for making a large fowl, comprising:

introducing a mutant $p27^{Kip1}$ gene lacking a Cdk2 phosphorylation site into a genome of a fowl cell by contacting *in vivo* a blastodermal cell of a fertilized cell with the mutant $p27^{Kip1}$ gene, wherein the $p27^{Kip1}$ gene is introduced directly into the germinal disk of the egg.

37.   The method of claim 36, wherein the fowl is a chicken, ostrich, emu, turkey, duck, goose, quail, parrot, parakeet, cockatoo or cockatiel.

SEQUENCE LISTING

<110> FRED HUTCHINSON CANCER RESEARCH CENTER

       MALEK, Nisar P.

       ROBERTS, James M.

<120> COMPOSITIONS AND METHODS FOR INCREASING ANIMAL SIZE AND GROWTH RATE

<130> 14538A-66-1PC

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<211> 48

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Ser Val Glu Gln Thr Pro Lys Lys Pro Gly Leu Arg Arg Arg Gln Thr
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<213> Homo sapiens

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Ser Val Glu Gln Thr Pro Lys Lys Pro Gly Leu Arg Arg Arg Gln Thr
            35   40   45

<210> 6

<211> 48

<212> PRT

<213> Homo sapiens

<400> 6

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            20   25   30

Ser Val Glu Gln Thr Pro Lys Lys Pro Gly Leu Arg Arg Arg Gln Thr
        35   40   45

<210> 7

<211> 48

<212> PRT

<213> Feline

<400> 7

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1   5   10   15

Ala Asn Arg Thr Glu Glu Asn Val Ser Asp Gly Ser Pro Asn Ala Gly
            20   25   30

Ser Val Glu Gln Thr Pro Lys Lys Pro Gly Leu Arg Arg Arg Gln Thr
        35   40   45

<210> 8

<211> 48

<212> PRT

<213> Porcine

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1   5   10   15

Ala Asn Arg Thr Glu Glu Asn Val Ser Asp Gly Ser Pro Asn Ser Ala
            20   25   30

Ser Val Glu Gln Thr Pro Lys Lys Pro Gly Leu Arg Arg Arg Gln Thr

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                35   40   45

<210>   9

<211>   46

<212>   PRT

<213>   Murine

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Met Arg Lys Arg Pro Ala Ala Glu Asp Ser Ser Ser Gln Asn Lys Arg
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Ala Asn Arg Thr Glu Glu Asn Val Ser Asp Gly Ser Pro Asn Ala Gly
                20   25   30

Thr Val Glu Gln Thr Pro Lys Lys Pro Gly Leu Arg Arg Gln
                35   40   45

<210>   10

<211>   48

<212>   PRT

<213>   Hamster

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Met Arg Lys Arg Pro Ala Ala Asp Asp Ser Ser Ser Gln Asn Lys Arg
1   5   10   15

Ala Asn Arg Thr Glu Glu Asn Val Ser Asp Gly Ser Leu Asn Ala Gly
                20   25   30

Ser Val Glu Gln Thr Pro Lys Lys Pro Gly Leu Arg Arg His Gln Thr
                35   40   45

<210>   11

<211>   46

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<213>   Ratus

<400>   11

Met Arg Lys Arg Pro Ala Ala Glu Asp Ser Ser Ser Gln Asn Lys Arg
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Ala Asn Arg Thr Glu Glu Asn Val Ser Asp Gly Ser Pro Asn Ala Gly
                                Page 4

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              20   25   30

Thr Val Glu Gln Thr Pro Lys Lys Pro Gly Leu Arg Arg Gln
        35   40   45

<210>   12

<211>   46

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<213>   Ratus

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            20   25   30

Thr Val Glu Gln Thr Pro Lys Lys Pro Gly Leu Arg Arg Gln
        35   40   45